Cell therapy in acute myocardial infarction

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Contents

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List of papers

I. Beitnes JO, Hopp E, Lunde K, Solheim S, Arnesen H, Brinchmann JE, Forfang K, Aakhus S. Long term results after intracoronary injection of autologous mononuclear bone marrow cells in acute myocardial infarction. The ASTAMI randomized, controlled study. *Heart.* 2009;95:1983-1989.

II. Beitnes JO, Gjesdal O, Lunde K, Solheim S, Edvardsen T, Arnesen H, Forfang K, Aakhus S. Left ventricular systolic and diastolic function improve after acute myocardial infarction treated with acute percutaneous coronary intervention, but are not influenced by intracoronary injection of autologous mononuclear bone marrow cells: a 3 year serial echocardiographic sub-study of the randomized-controlled ASTAMI study. *Eur J Echocardiogr.* 2011;12:98-106.

III. Beitnes JO, Øie E, Shahdadfar A, Karlsen T, Müller RMB, Aakhus S, Reinholt FP, Brinchmann JE. Intramyocardial injections of human mesenchymal stem cells following acute myocardial infarction modulate scar formation and improve left ventricular function. *Cell Transplantation. In press.*

Abbreviations

ACE	angiotensin converting enzyme
ADSC	adipose tissue derived stem cells
AMI	acute myocardial infarction
ARB	angiotensin receptor blocker
ASTAMI	autologous stem cell transplantation in acute myocardial infarction
BNP	brain natriuretic peptide
BM	bone marrow
BOOST	bone marrow transfer to enhance ST-elevation infarct regeneration
CABG	coronary artery bypass grafting
CD	cluster of differentiation
CHF	congestive heart failure
CPCs	cardiac progenitor cells
CRF	case report form
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DSMB	data safety and monitoring board
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
EHS	European heart survey
EPCs	endothelial progenitor cells
ESCs	embryonic stem cells
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FISH	fluorescence in situ hybridization
FS	fractional shortening
GFP	green fluorescent protein
GMP	good manufacturing practise
HBSS	Hank's balanced salt solution
HIV	human immunodeficiency virus
ICD	implantable cardioverter defibrillator
iPS	induced pluripotent stem cells
JOB	Jan Otto Beitnes

IRA	infarct related artery
KL	Ketil Lunde
LAD	left anterior descending (coronary) artery
LCX	left circumflex (coronary artery)
LPRF	low pulse repetition frequency
LV	left ventricle/ left ventricular
LVEF	left ventricular ejection fraction
LVEDV	left ventricular end diastolic volume
LVESV	left ventricular end systolic volume
LVEDd	left ventricular end diastolic diameter
LVESd	left ventricular end systolic diameter
MACE	major adverse cardiovascular event
MAGIC	myoblast autologous grafting in ischemic cardiomyopathy
mBMC	mononuclear bone marrow cells
MHC	major histocompatibility complex
mPBC	mononuclear peripheral blood cell
MRI	magnetic resonance imaging
MSC	mesenchymal stem cell
NA	not available
NiH	National Institute of Health
Nt-proBNP	N-terminal pro-brain atriuretic peptide
OG	Ola Gjesdal
OUES	oxygen uptake efficiency slope
OUS	Oslo University Hospital
PCI	percutaneous coronary intervention
PCR	polymerase chain reaction
pCO_2	partial pressure of carbon dioxide
pO_2	partial pressure of oxygen
QoL	quality of life
RBCs	red blood cells
RCA	right coronary artery
REGENT	myocardial regeneration by intracoronary infusion of selected population of stem cells in acyute mycadial infarction

REPAIR-AMI	reiinfusion of enriched progenitor cells and infarct remodeling in acute myocardial infarction
RER	respiratory exchange ratio
RH	Rikshospitalet University Hospital
RNA	riboxy nucleic acid
SD	standard deviation
SMA	small muscle actin
SM- MSC	skeletal muscle derived mesenchymal stem cells
SPECT	single photon emission computed tomography
STEMI	ST- elevation myocardial infarction
SVF	stromal vascular fraction
TIMI	thrombolysis in myocardial infarction
TNF-α	tumor necrosis factor alpha
UiO	University of Oslo
UUS	Ullevål University Hospital
VE	ventilation
VO_2	oxygen consumption
VCO_2	carbon monoxide production
WBCs	white blood cells
WHO	World Health Organization
WMSI	wall motion score index
2D-STE	2 dimensional strain echocardiography

Introduction

In acute myocardial infarction (AMI), ischemia initiate the necrosis and apoptosis of millions or even more than a billion cardiomyocytes.[1] After weeks of inflammation and reparative processes, these cells are largely replaced by scar tissue. Substantial loss of functional myocardium leads to acute heart failure, but even moderate infarcts may increase the strain/stress on remaining cardiomyocytes, and thereby induce a maladaptive compensatory response leading to congestive heart failure (CHF). This change in myocardial structure and left ventricular (LV) geometry, often referred to as remodeling, may act beneficially in the shorter term to increase stroke volume, but is in the long run associated with a poor outcome.[2] Ischemic heart disease and congestive heart failure are important causes of morbidity and death in industrialized countries.[3]

The pathophysiology in CHF is incompletely understood. Through history, different pathophysiological models have each made important intellectual and therapeutic contributions. The cardiorenal model implemented the use of diuretics, vasodilatators and inotropic drugs, alleviating oedemas and releaving symptoms, but with dubious prognostic effects. Later, the neurohormonal model introduced angiotensin converting enzyme (ACE)-inhibitors, betablockers, angiotensin receptor blockers (ARBs) and aldosterone-antagonists. In large, randomized-controlled trials, their effects on morbidity and mortality were well documented through the 1980s and -90s, and these drugs are still cornerstones in treatment in CHF and after AMI.[4, 5] At the end of the 90s, a cardioinflammatory model was introduced, clarifying important steps in the pathophysiology of CHF. New potential targets for therapeutic interventions were revealed, but in clinical trials, most of these interventions have failed to prove clinically effective (i.e. TNF-α antagonists[6], BNP (Nesiritide®)[7], and immunoglobulins[8]). In end–stage heart failure, heart transplantations or ventricular assist devices may save lives in selected groups of patients. However, the availability of donor organ is limited, and patients are exposed to the risks for rejection and complications of high-intensity immunosuppression. The ventricular assist devices have improved with the introduction of non-pulsatile rotations pumps, but this therapy is still expensive with high risk for infections and thromboembolic events. Thus, mortality in post-infarction CHF remains high, and new therapeutic options are needed.

The heart as a self-renewing organ

The heart has for centuries been regarded a terminally differentiated organ without regenerative capacity after birth. Mitotic figures in cardiomyocytes were rarely identified in the light microscope, and in response to injury, contractile cells were replaced by scar tissue. Even in the process of cardiac hypertrophy, cells seemed to increase in volume, and not in number.[9, 10] In the last decade, this paradigm has been challenged. A cardiac stem cell has been identified, and proliferative activity has been documented.[11] Bergmann et al. have, with analyses of the C^{14} isotope, provided evidence that the heart is self-renewing, but at a slow rate.[12] Kajstura et al. suggest a more rapid turnover of cardiomyocytes, based on histological staining for protein markers of cell division (Ki67 and phospho-H3) and apoptotic cell death ($p16^{INK4a}$).[13] However, clinical experience tells us that the hearts intrinsic regenerative capacity is insufficient when challenged by substantial myocardial damage like AMI, myocarditis, or cardiotoxic drug therapy.

Cell therapy

Bone marrow transplantations have been performed successfully since 1959 for hematological or malignant disorders, showing that allogeneic cells may reconstitute bone marrow function in the recipient. The stem cells themselves (colony forming units) were not identified until 1963, but their role in the hematopoietic system has been well described.[14, 15] Stem cells are defined by 3 important characteristics;

- self-renewal
- potency- the ability to differentiate to specialized cell types and
- the ability to reconstitute a tissue.

Thus, stem cells may undergo symmetric division to provide two stem cells or two differentiated daughter cells, or asymmetric cell division to provide one stem cell and one differentiated daughter cell. Stem cells have been classified according to their potential for differentiation:

- Totipotent stem cells= the fertilized oocyte and descendants until the 8-cell stage of embryonic development.
- Pluripotent stem cells= stem cells able to differentiate to all three germ layers (endo-, ecto-, and mesoderm), i.e. embryonic stem cells (ESCs).

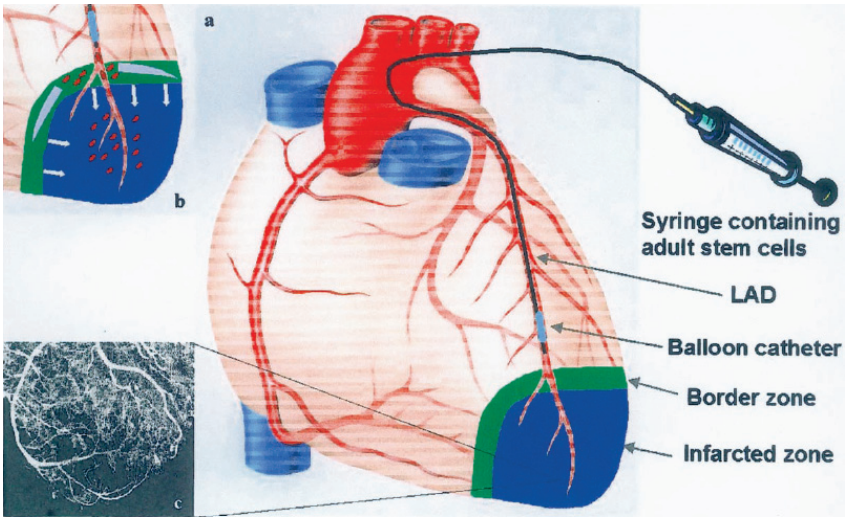
- Multipotent stem cells= stem cells able to differentiate to > 1 lineage of daughter cells. Mesenchymal stem cells and hematopoietic stem cells are examples of multipotent stem cells.
- Unipotent stem cells= cells capable of differentiation to one cell type. Also called progenitor cells. Endothelial progenitor cells (EPCs), cardiac stem cells (CSCs) and skeletal myoblasts are all examples of unipotent stem cells.

Stem cells have been identified in most organs, including the heart, suggesting a potential for regeneration of lost cells. Stimulation of endogenous stem cells- either circulating or resident *in situ*, direct transplantation of stem cells, and tissue engineering are the most important approaches in this field of therapy, often referred to as regenerative medicine. Repopulating the heart with functional cardiomyocytes could, in theory, be a causal and definite therapy after larger a large AMI or in CHF. Different approaches have been explored experimentally and clinically, as methodology and experience have progressed. In the early 1990s, skeletal myoblasts, also called satellite cells, were the first cells used.[16] These cells were striated and contractile like cardiomyocytes, but easier to harvest and culture. In experimental models, implanted cells were readily identifiable in the host myocardium, remodeling was abrogated, and LV function improved.[17] Unfortunately, further studies revealed that the transplanted myoblasts did not couple electrically with the host myocardium.[18] In clinical trials, increased risk of arrhythmias was observed, and implantable cardioverter-defibrillators (ICDs) had to be implanted in all recipients. In the first clinical phase II trial, MAGIC[19], the increased burden of ventricular arrhythmias was confirmed, and no significant benefit was found on the primary endpoint LV ejection fraction (LVEF). This discouraging result practically terminated further clinical research on cardiac myoblast therapy.

In 2001, Orlic et al. reported substantial engraftment, myocardial differentiation and improvement in LV function when bone marrow cells (BMCs) were injected into the heart after myocardial infarction in mice.[20] Although the results evoked scientific controversy[1, 21, 22], the use of BMCs was rapidly translated directly from small animals to human trials. Already in 2002, Strauer et al. presented a cohort-study on patients treated with intracoronary infusion of bone marrow cells after AMI.[23] The mononuclear BMCs (mBMCs) had been isolated by gradient centrifugation in a polysaccharide suspension (Ficoll) and were subsequently infused in the infarct related coronary artery (IRA) through a percutaneous coronary intervention (PCI)- catheter (figure 1). The PCI- balloon was inflated

at the site of former coronary occlusion to stop flow for 2-4 minutes while 2-3 ml of cell-suspension was infused, to allow time for cells to adhere to and migrate through the vessel wall. Then, the balloon was deflated to allow blood-flow for a few minutes and the procedure was repeated 6 to 7 times to infuse all cells.

Figure 1. Intracoronary cell- injection by stop-flow technique.



B Strauer et al. Circulation 2002:106;1913-1918. Used with permission from the publisher.

In Strauer's hands, the method appeared safe and patients treated with mBMC injections had decreased infarct size and improved regional LV function after 3 months when compared to the control group. Patients who refused participation in the cell therapy group served as controls, thus, this was not a randomized study. However, the results were encouraging and the "stop-flow" technique was adopted by subsequent clinical trials. Although the mechanisms of action, dose-response relationship, best route of administration, and optimal timing of cell therapy were still unclear, several small or medium sized randomized studies were initiated to further explore the potential of this new treatment. The Norwegian ASTAMI study was initiated in 2003, and the primary results were published in 2006.[24] The trial design and results after 6- and 12 months have been presented and discussed in previous publications and the dissertations by Ketil Lunde and Svein Solheim.[24-28] In ASTAMI, mBMC therapy did not improve LVEF after 6 months compared to the control group. In contrast, the contemporary REPAIR- AMI study showed significant improvement in LVEF 4 months after cell therapy.[29] These apparently

conflicting results evoked a controversy on cell preparation procedures.[30-33] However, both studies included a limited number of patients, without power to determine effects on clinical outcome. The results of most small and medium sized trials on mBMC therapy have been summarized in several meta-analyses[34-36] and a schematic overview of studies with ≥100 patients and/or long term follow-up data are presented in table 1. Based on data in these meta-analyses, mBMC-therapy after AMI is considered safe. Although PCI procedures are potentially harmful, and possible side effects of bone marrow aspirations and PCI procedures have been reported[37-39], adverse events are not overrepresented in the cell treated patients. The pooled data in the Cochrane-based meta-analysis[34] suggest a modest beneficial treatment -effect on LVEF of 3 % (percentage-points) after 4-6 months of observation. However, experimental data show that the engraftment of injected cells is poor[40-42], and that differentiation of bone marrow cells to cardiomyocytes after injection hardly occurs.[21, 22] Thus, after these early trials a move "back to the bench" was suggested- to search for more efficient cells and to clarify mechanisms of action, optimal cell dosing, timing and route of cell administration.[43] Clinical data from long term follow-up of patients treated with mBMC therapy were also requested[34, 43] The treatment modality was only recently introduced, patients in most studies had been followed for only 4-6 months, and beneficial effects were discrepant. Thus, it was imperative to obtain long term follow- data on safety and efficacy in patients from the ASTAMI trial, one of the largest trials in this field. Based on these considerations, we performed a 3 year follow-up of patients in the ASTAMI- trial, and initiated a preclinical project to investigate effects of other cells. In 2006, Norwegian legislation did not permit research on ESCs. Results in clinical studies with myoblasts and mBMCs were rather disappointing. In the population in need of cardiac cell therapy, autologous CPCs were considered very rare and difficult to culture (personal communication, prof. Philippe Menasche, Paris, France). Mesenchymal stem cells (MSCs) constitute a small fraction (0.001-0.01 %) of BMCs and were described by Pittenger et al in 1999.[44] They are easily cultured, and characterized in culture by adherence to plastic surfaces. By definition, these cells have multilineage potential, forming fat, cartilage or bone when cultured in conditioned media. In specific conditions, the cells have also been stimulated to express markers of endothelial-, smooth muscle- and even cardiomyocyte phenotypes in culture.[45, 46] Like the skeletal myoblasts, MSCs are relatively large and adhesive cells that may cause vascular plugging[47], and large numbers of cells are therefore rarely infused in systemic arteries. Cells have been administered either by catheter-based transendocardial injections, as i.v. infusions, or as direct intramyocardial injections during

surgery.[48-50] The majority of MSCs administered i.v. will be trapped in the pulmonary circulation.[51] Early experimental studies reported improved LV function, reduced infarct size and improved vascular density after MSC injections.[45, 50, 52-54] The mechanisms of action were, however, not clarified. Cells with an MSC phenotype had also been isolated from other, more easily available tissues like fat (ADSCs) and skeletal muscle.[55] The potential for myocardial regeneration by use of these new cell lines was unknown, and head-to-head comparisons of the effects of MSCs from different tissues had not been published. Availability, expandability, the theoretical potential for differentiation, and existing experimental results suggested MSCs as an attractive potential cell type for cardiac regeneration. We therefore sought to establish an animal model to test these cells by intramyocardial injection after AMI. The ASTAMI long term follow- up and the experimental studies on adipose tissue- and skeletal muscle derived stem cells were initiated in 2007 as a PhD- project.

Table 1. Randomized controlled studies on cardiac BMC therapy in AMI. [#]

Name of study, principal author, year	Size	BM volume and dose (x 10^6 cells)	Preparation	Timing (days after AMI)	Primary endpoint	Follow-up	Baseline EF	ΔEF (% points)	Δ LV EDV (ml)	Δ Infarct size (MRI)	Other effects reported
BOOST (Wollert, 2004)	30 mBMC 30 Control	50 mL 2460±940	Succinate-gel	4.8±1.3	LVEF by MRI	6 months	50.0±10.0 51.3±9.3	P=0.003 6.7±6.5 0.7±8.1	7.6±20.0 3.4±11.1	-14.1±13.0 ml -10.5±10.6 ml	Improved diastolic function by echo in the BMC group (not predefined endpoint)
BOOST (Meyer, 2005)					LVEF by MRI	18 months		P=0,27 5.9±8.9 3.1±9.6	6.1±20.3 3.6±15.1	-12.8±11.8 ml -10.1±13.1 ml	
BOOST (Meyer, 2008)					LVEF by MRI	60 months		P=0.30 -2.5±11.9 -3.3±9.5	13.9±25.4 7.5±17.3	-15.0±12.1 ml -14.6±12.1 ml	
ASTAMI (Lunde, 2006)	50 mBMC 50 Control	50 mL 68 (54-130)	Lympho-prep	6 (5-6)	LVEF by SPECT	6 months	41,3±10,4 42.6±11.7	P=0.77 8.1±11.2 7.0±9.6	-11.2±36.0 -1.8±17.6	-2.4±10.9ml -5.8±13.5ml*	Improved exercise time in mBMC group (not predefined endpoint)
ASTAMI (Beitnes, 2009)					LVEF by echo	36 months	45.7±9,4 46.9±9.6	P=0,28 2.2±7.3 -0.4±8.2	P=0.77 2.1±34.9 6.3±35.8	-11.4±11.4 ml -13.8±17.0 ml*	
REPAIR-AMI (Schächinger, 2006)	101 mBMC 103 placebo	50 mL 236±174	Ficoll-Hypaque, Cambrex	4.3±1.3	LVEF by ventriculography	4 months	48.3±9.2 46.9±10.4	P=0,01 5.5±7.3 3.0±6.5	P=0.64 12±31 14±33	NA	MACE reduced in mBMC group (not predefined endpoint)
REGENT (Tendera, 2009)	80 mBMC 80 SEL 40 Control	mBMC: 50-70 mL 178x 10^6 SEL: 100-120 mL 1.9 x 10^6	Ficoll-Hypaque, Cambrex	7 (3-12)	LVEF by MRI	6 months	37 (19-44) 35 (12-45) 39 (23-44)	P=0,19 3 % 3% 0%	P=ns 10 13 -3 †	NA	
HEBE (Hirsch, 2010)	69 (ic) mBMC 66 (ic) mPBC 65 Control	mBMC: 60 mL 296±164 mPBC: 150-200 mL 287±137	Lympho-prep	mBMC: 6 (4-7) mPBC: 5 (4-6)	Percentage of improved dysfunctional LV segments by MRI	4 months	43.7±9,0 41.7±9,1 42.4±8.3	P=0.90 3.8±7.4 4.2±6.2 4.0±5.8	P=0,33 5.4±13.4 5.3±16.3 8.2±13.5	-7.7±8.5g -7.9±6.5g -9.4±7.1g	Trend for negative effekt of cell therapy (P= 0,14) on primary enpoint

#Studies with ≥100 included patients and/or ≥1 years follow-up are presented. NA= not available. BM= bone-marrow. SEL= $CD34^+CXCR4^+$ mBMCs. mPBC= mononuclear peripheral blood cells. Numbers are mean±SD or median (25th percentile-75thpercentile). Ns=not significant. *Baseline MRI performed 2 weeks after inclusion/ mBMC therapy.

† Change in median, based on supplementary data.

Aims of the thesis

The aims of the present work were:

1. To perform a follow-up of patients in the ASTAMI study 3 years after randomization for evaluation of long term efficacy and safety, including recording of clinical events, physical examination, the SF-36 quality-of-life survey, blood biochemistry analyses, echocardiography, exercise testing, and cardiac MRI (paper I).

2. To perform a comprehensive analysis of all echocardiographic recordings from baseline to 3 years follow-up in patients from the ASTAMI study, including systolic, diastolic and regional function (paper II).

3. To establish an experimental model for cell therapy in acute myocardial infarction.

4. To evaluate the effects of mesenchymal stem cells (MSCs) harvested from skeletal muscle and adipose tissue in a translational model of intramyocardial cell injection in AMI (paper III).

Subjects and methods (clinical study)

Patients

The ASTAMI study was an open-labeled, randomized controlled clinical trial. All patients admitted to Rikshospitalet University Hospital (RH) and Ullevål University Hospital (UUS) in the period September 2003 to May 2005 for acute PCI due to ST-segment elevation myocardial infarction (STEMI) were screened for inclusion (n=1608).

Inclusion criteria (all criteria had to be fulfilled):

- Age between 40 and 75 years
- Acute anterior wall myocardial infarction with a history >3 and <12 hours
- ST-elevation infarction (WHO ECG criteria)
- Angiographic criteria: Occlusion (TIMI flow 0-1) of the left anterior descending (LAD) coronary artery proximal to the second diagonal branch.
- Successful primary or rescue PCI (TIMI flow 2 or better)
- Echocardiographic criteria: Evidence for anterior wall infarction as judged by hypo- or akinesia in more than 2 adjacent anterior wall segments.
- Enzymatic criteria: CK-MB mass >3 times upper normal value

Exclusion criteria:

- Previous myocardial infarction with established significant Q-waves on ECG.
- Cardiogenic shock.
- hemodynamic instability necessitating intraaortic balloon pump treatment.
- Permanent pacemaker or other contraindications to magnetic resonance imaging (MRI).
- Stroke with significant sequela.
- Short life expectancy due to extra cardiac reason, i.e. chronic obstructive lung disease, disseminated malignant disease, or other reason.
- Uncontrolled endocrinological disturbance.
- Anamnestic indications for significant mental disorder, including dementia.
- Established HIV or hepatitis B infection.
- Any condition which interferes with the patients' possibility to comply with the protocol.

After written, informed consent, study participants were randomized 1:1 to intracoronary injection of autologous mBMCs 4 to 8 days after the acute event (n=50), or to a control group where neither bone marrow aspiration nor sham coronary procedure were performed (n=50). Three patients allocated to the mBMC group did not receive intracoronary cell injections (due to stent-thrombosis preceding the cell infusion in two patients, cell viability <90 % in one patient).

The primary endpoint in ASTAMI was the change in LVEF from baseline to 6 months measured by single photon emission computed tomography (SPECT). MRI and echocardiography were used for serial assessment of LV function. Secondary endpoints were changes in exercise capacity and quality-of-life (QoL). Clinical events, infarct size, and multiple inflammatory and other biochemical markers were also evaluated. An echocardiogram and evaluation of clinical status after 12 months finished the initial protocol. The main results from the first 12 months of follow-up have been presented previously.[24-28] The follow-up after 3 years was not part of the original study protocol. Thus, a new study protocol was written and approved by the regional committee for ethics in medical research. Patients provided a new written, informed consent. At 3 years, one patent refused further clinical follow-up and 2 patients were dead. Thus, 97 patients were eligible for the extended study. Medical history, clinical examination, SF-36 QoL-questionnaire, exercise testing, transthoracic echocardiography and cardiac MRI were performed. To minimize patient hazard, SPECT and routine coronary angiography were not performed at 3 years.

Cell processing and injection

From patients in the mBMC group, 49±9 mL (mean±SD) bone marrow in 10.000 IU heparin was harvested from the iliac crest 4 to 7 days after AMI. The aspirate was diluted in 75 ml 0.9% saline, and the mononuclear cell fraction was isolated by Isopaque-Ficoll (Lymphoprep©, Axis-Shield, Oslo Norway) gradient centrifugation. The mBMCs were washed 3 times, resuspended in 11 mL 0.9 % saline with 20 % heparin-plasma and stored over night. Median cell viability, as assessed by an acridin orange/ethidium bromide exclusion assay, was 95 %. The median number of injected viable cells was 68 x 10^6 (interquartile range 54x10^6 to 130x10^6). The cell products were processed in an accredited cell production facility under Good Manufacturing Practice (GMP) conditions. Approximately 10 mL of cell suspension was injected by PCI on LAD with the stop flow technique 5 to 8 days after the AMI. One third of the cell suspension was injected during 90

s balloon inflation and no-flow, followed by 5 min. reperfusion, and this procedure was repeated twice.

Cardiac MRI

Patients without contraindications underwent MRI 2-3 weeks (n=89), 6 months (n=93), and 3 years (n= 88) after randomization. 1.5 T scanners were used (Magnetom Vision Plus (Siemens, Erlangen, Germany) for the first 18 months of ASTAMI, then Magnetom Sonata (Siemens) for the last 10 months of ASTAMI and the 3 year follow-up). Breath-hold cine-sequences were obtained in 2-chamber, 4-chamber, and short-axis views with ECG-triggered segmented gradient echo techniques for calculation of LV volumes and LVEF.

LVEF = (EDV-ESV) / EDV

Two different image processing protocols were used: Compact-FLASH (n=63) and true FISP (n=24, available with Magnetom Sonata, only). To avoid bias in serial evaluation of volumes, the same processing method was used for all acquisitions in each patient. LVEDV, LVESV, and LVEF were calculated by the biplane area-length method;

$V = (8 \times A_1 \times A_2) / (3 \times \pi \times L)$.[56, 57]

Where V=volume, L=length (longest axis), A_1 and A_2 are the traced endocavitary areas.

At 3 years, for comparison of methods, a stack of multiple short axis cine images were also recorded to calculate LV volumes by the Simpson method.[56]

$$\text{Volume} = h \sum_{x=1}^{n-1} (A_x) + hA_n/2 + h^3(\pi/6)$$

Where h= distance between centers of adjacent slices, n=number of slices, A_1=area of the most basal slice, and A_n=area of the most apical slice.

LV mass was determined from a stack of short axis images covering the entire LV (slice thickness 7 mm, interslice gap 3 mm). Infarct size was determined from the short axis images 10-20 minutes after iv. injection of 0.2 mmol/kg gadopentetate dimeglumine (Magnevist®, Schering AG, Berlin, Germany) contrast medium. For this late enhancement contrast imaging, slice thickness was 8 mm, increment 10 mm, and in-plane spatial resolution was 1.17 x 1.17 x 1.5 mm. Myocardial- and infarct masses were calculated by multiplying volume with the tissue density 1.05 g/mL.[58] A more detailed description of the

MRI processing and analyses can be found in the publication by Hopp et al. on a subgroup of patients from ASTAMI.[59]

Echocardiography

Transthoracic echocardiography was performed at baseline (4.5±1.1 days after AMI) and 3, 6, 12, and 36 months after randomization, using a high-end cardiac ultrasound scanner (Vivid 7 with the M3S transducer, GE Vingmed, Horten, Norway). The recordings and analyses were performed according to general recommendations.[60-62] LV volumes and ejection fraction (LVEF) were calculated by the modified Simpson method, tracing endocardial contours on apical 4-chamber and apical long-axis images. When 2 chamber images were of clearly better quality than the long-axis images, these were used.[63] Tissue Doppler recordings were obtained from the apical 4-chamber view. Peak early diastolic (e') velocities were registered in the septal and lateral mitral annulus, and mean values used for further calculations. Wall motion score was assessed by use of a segmental LV model, excluding the apical cap. All 16 segments were assigned a wall motion score (1=normal or hyperkinetic, 2=hypokinetic, 3=akinetic, 4=dyskinetic, and 5=aneurysmatic). The sum of scores was divided by the number of segments visualized, to obtain the wall motion score index (WMSI). Two-dimensional speckle tracking echocardiography (2D-STE) was performed in the standard 3 image planes acquired from the apex. Longitudinal (peak negative systolic) strain was measured to assess regional and global myocardial function in a 16 segment model of the left ventricle.[64] As all patients in ASTAMI had LAD- related infarcts, the infarct zone was defined as the six segments schematically supplied by LAD. All other segments, schematically supplied by ramus circumflexus (LCX) and the right coronary artery (RCA), were defined as remote. Regional- and global strain were calculated as the average of all analyzed corresponding segmental strain values.[65] Peak E- and A-wave velocities, and E-wave velocity deceleration time (DT) were measured, and E/A-ratio was calculated. Mitral A-wave duration was measured from flow recordings at the level of the mitral valve. Flow signals in the right upper pulmonary vein (pv) were obtained by low pulse repetition frequency (LPRF) Doppler from the 4 chamber apical view. Peak systolic (pvS)-, -diastolic (pvD)-, and -atrial reverse (pvA)- wave velocities and pvA wave duration were recorded, and the pvS/pvD-ratio was calculated. E/e'-ratio was calculated from peak early mitral inflow velocity (E) and peak early mitral annulus velocity (e') in the apical 4 chamber view. Except one patient with atrial fibrillation during the baseline recording, all patients were in sinus rhythm at all examinations. One patient in the control group had right-

bundle branch block from baseline. One patient in the mBMC group developed left bundle branch block between 12 months and 3 years after inclusion. All analyses were performed offline on GE Echopac software, blinded to patient identity and treatment allocation.

Cardiopulmonary exercise test

Maximal symptom limited exercise tests were performed 2-3 weeks, 6 months, and 3 years after the AMI with an electrically braked bicycle ergometer (Jaeger ER900, VIASYS Healthcare GmbH, Hochberg, Germany). At 6 months and 3 years, betablockers, calcium channel blockers, and nitrates were postponed until after the test. Other medication was taken as usual. 10 minutes exercise time was pursued, starting at 25 or 50 W with 10 or 25 W increase every second minute. For each patient the same protocol was used for every test. Oxygen consumption (VO_2), CO_2 production (VCO_2), and ventilation (VE) were measured on a breath-to-breath basis (MVmax 229, VIASYS Healthcare GmbH, Germany). Heart rate and ECG were continuously recorded. Blood pressure was measured at the end of each step. For parameters sampled continuously or breath to breath, average values from 20 second time intervals were used in the analyses. Maximal VO_2 and respiratory exchange ratio (RER) was measured in the last 20-second interval during exercise. The anaerobic threshold was defined as the VO_2 at the point where ventilation started to increase exponentially compared to the increase in VO_2. This point was determined by the V-slope method (using the VCO_2/VO_2-plot) whenever possible, and supplied by use of the VE/VO_2-plot and the plot of end-tidal pO_2 and end-tidal pCO_2.[66] The VE/VCO_2-slope was determined by a linear regression analysis of all data obtained in the the VE/VCO_2-plot during exercise.[67] The MVmax 229 software was used for calculation. The oxygen uptake efficiency slope (OUES)[68] was calculated by data from the second 20 –second time interval after start of exercise, data sampled at AT, and data at VO_2 max. The slope was calculated as the best fit linear regression line relating $\log_{10}VE$ and VO_2 by use of Excel software (Microsoft, Redmond, WA).

Blood biochemistry analyses

Blood samples were collected by standard venipuncture between 8 and 9 AM after a 12-hour fast. Serum was prepared by centrifugation within 1 hour at 2500 x g for 10 minutes. All blood biochemistry analyses in the 3 years follow-up were performed mainly for monitoring of safety, and were analyzed as routine samples at Department of Medical Biochemistry, Rikshospitalet.

Quality of life

For assessment of health-related QoL, patients answered the Norwegian version 1.2 of the SF-36 health survey 2-3 weeks, 6 months, and 3 years after AMI. Scores were weighted and aggregated using normative data from the general Norwegian population to obtain physical component- and mental component summary scores. The calculations of weighted scores were performed by Torbjørn Moum, who also provided the normative data.

Statistics

Continuous data with approximated normal distribution were presented as mean±standard deviation. Independent sample t-tests were used to compare the groups at baseline. For analysis of continuous data measured at ≥3 points of time, we used mixed model linear regression analysis. Time and the interaction between treatment allocation and time were covariates. Treatment allocation was removed as a factor to adjust for differences between groups at baseline. Intragroup changes from baseline were evaluated by paired sample t-tests. Continuous data with skewed distribution were presented as median (interquartile range), and Mann-Whitney tests were used for comparison between groups. Categorical data were presented as frequency (percentage) and Chi-Square or Fisher's exact tests were used as appropriate. EpiData entry software version 3.1 and SPSS version 15.0 were utilized. All analyses were performed according to the intention-to-treat principle, tests were two-sided, and p-values <0.05 were considered statistically significant.

Ethics

The protocol was approved by the regional committee for research ethics and the study complies with the Declaration of Helsinki All patients gave written, informed consent. The ASTAMI study is registered at clinicaltrials.gov NCT 00199823.

Model, materials and methods

(experimental study)

Animals

For an experimental model of stem cell therapy in acute or recent MI, several animal models can be considered. For the present study, the Institute of Immunology (IMMI) had well charachterized human MSCs available, thus a xenogenic model was needed to allow injection of human cells into animal hearts. Xenoimmune rejection of transplanted cells could jeopardize the potential for long term engraftment of transplanted cells. Immunodeficient strains of rats and mice are available, while concomitant immunosuppressive pharmacotherapy would be required if immunocompetent strains of rats or mice, or a larger animal like rabbit, dog or pig were used. The dosing of immunosuppressants would be unreliable if administered by oral feeding, and would be a significant stressor if administered by daily injections. Immunosuppressants would also potentially confound the results by modulation of the inflammatory response and/or remodelling after AMI, as described for calcineurin inhibitors.[69] Mice are small, and the risk of unsuccessful myocardial cell-injection has been reported as high as 50 %.[20] Based on these considerations, NiH rnu/rnu nude rats were used. The Rowett rnu-gene was a spontaneous mutation leading to a hairless, athymic, T-cell deficient phenotype in homozygous rats. The NiH rnu/rnu rats were developed in 1979-80 by adding and backcrossing the Rowett rnu-gene into eight inbred rat strains. Both Rowett and NiH rnu/rnu rats are in well established use for research in xenotransplantation.[70, 71] The animals may acquire some cellular immunity with age, and it has been suggesteded that xenotransplantation should be performed before the age of 12 weeks to minimize risk of rejection. In our study, male NiH rnu/rnu rats and female rnu/+ rats (Charles-River, France) were bred at the Rikshospitalet animal facility by experienced staff, and rnu/rnu progeny were selected for use in our studies. Animals were maintained in a specific pathogen-free environment in positive pressure rooms with a standard 12 hour day/12 hour night cycle, 2-4 animals per cage, with environmental enrichment. The animal department is subject to

regular microbiological testing. No significant pathogens were encountered in the rnu-rats during the period for our experiments.

Cell processing

A more detailed description of cell processing is presented in paper III.

Isolation and culture of human skeletal muscle-derived MSCs (SM-MSCs)

Skeletal muscle tissue was obtained from the gracilis and semitendinosus muscles removed from patients undergoing surgery of the anterior cruciate ligament. The donors provided written, informed consent, and the collection and storage of muscle tissue and SM-MSCs was approved by the regional committee for ethics in medical research. Ten to 20 g of skeletal muscle was washed, minced and digested in collagenase IA (Sigma), and resuspended in trypsin- EDTA (Sigma). The isolated cells were washed and subsequently cultured in DMEM/F12 containing 20% FBS, amphotericin B and antibiotics. Muscle cells were expanded up to P5 for two donors and P7 for one donor. CD56+ cells were removed using magnetic beads directly coupled to mouse anti-human CD56 monoclonal antibody (MAb) (Miltenyi Biotech, Bergisch Gladbach, Germany) and LS columns, leaving a CD56- population which has previously been described as MSCs.[55] Cell viability was always >90%. Flow cytometry showed that no more than 3% of CD56+ cells were left in the suspension at P2 and last passage.

Isolation and culture of human adipose tissue-derived MSCs (ADSCs)

Adipose tissue (AT) was obtained as part of routine liposuction procedures from healthy donors. The donors provided written, informed consent, and the collection and storage of AT and ADSCs was approved by the regional committee for ethics in medical research. Lipoaspirate (300–1000 mL) was washed repeatedly with Hanks' balanced salt solution (HBSS) (Life Technologies-BRL, Paisley, UK), and digested using 0.1% collagenase A type 1 (Sigma). After centrifugation, floating adipocytes were removed. The remaining SVF cells were resuspended in HBSS containing 2% FBS. Suspended cells were filtered through cell sieves (Becton Dickinson, San Jose, CA) and layered onto Lymphoprep gradient separation medium (Axis Shield, Oslo, Norway). After centrifugation, cells at the gradient interface were collected, and resuspended. ADSCs were separated from the remaining cells using magnetic cell sorting. Endothelial cells ($CD31^+$) and leukocytes ($CD45^+$) were removed using magnetic beads directly coupled to mouse anti-human CD31 and CD45 Mab

(Miltenyi Biotech) and LS columns. Flow cytometry showed that no more than 5% of CD31+ and CD45+ cells were left in the suspension. Cells were washed, resuspended and seeded in DMEM/F12 containing 20% FBS and antibiotics. ADSCs from 3 donors were expanded to P5. Prior to injection, cell viability was always >90%.

Verification of the cells as MSCs by differentiation and flow cytometry studies

To verify that the cultured cells were actually MSCs, SM-MSCs and ADSCs were differentiated along adipogenic and osteogenic lineages and analyzed by flow cytometry for MSC markers. For flow cytometric analysis, cells were incubated with directly conjugated antigen specific or irrelevant monoclonal antibodies (Mabs), and fixed in 1% paraformaldehyde. Mabs used were CD56/PE, CD105/APC, CD44/PE, HLA ABC/Cy-Chrome, CD34/FITC, CD45/FITC (all BD Biosciences, CA), CD105/APC and HLA DR/APC (Diatec, Oslo, Norway), 144/PE (eBioscience, http://www.ebioscience.com/) and CD146/FITC (AbD Serotec, Kidlington, UK). Cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson).

For adipogenic differentiation, confluent cultures were incubated in DMEM/F12 containing 10% FBS, 0.5 µM 1-methyl-3 isobutylxanthine, 1 µM dexamethasone, 10 µg/mL insulin (Novo Nordisk, Copenhagen, Denmark), and 100 µM indomethacin (Dumex-Alpharma, Copenhagen, Denmark).

For osteogenic differentiation, cells were incubated at 3,000 cells per cm^2 in DMEM/F12 containing 10% AS or FBS, 100 nM dexamethasone, 10 mM ß-glycerophosphate, and 0.05 mM L-ascorbic acid-2-phosphate.

After 4 weeks, differentiated cells were examined by real-time (RT)-PCR and staining procedures. For real-time RT-PCR, total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA). Following DNase treatment (Ambion, Austin, TX), RNA was quantified by spectrophotometry (Nanodrop, Wilmington, DE). Reverse transcription (RT) was performed using the High Capacity cDNA Archive Kit (Applied Biosystems, Abingdon U.K.) with 200 ng total RNA per 20 µL RT reaction. Relative quantification (RQ) was performed using the 7300 Real-Time RT PCR system (Applied Biosystems) and Taqman® Gene Expression assays. All samples were scaled relative to the expression level of GAPDH (Taqman assay no Hs99999905_m1). For staining procedures, cells were fixed with 4%

formalin and subsequently incubated for 10 min with Oil-Red O and Alizarin Red S to visualize lipid droplets and calcium deposition, respectively.

At the day of surgery and cell injection, cells were centrifuged, washed, and resuspended in DMEM with 10 % FBS at $20x10^6$ cells/mL ($3x10^6$ cells/150µL). The cell suspension was stored on ice during transfer to the animal facility and injected within 2 hours. Immediately before injection, cells were resuspended, aspirated into a 300 µL syringe and warmed to approximately 37° C.

Surgery and cell injections

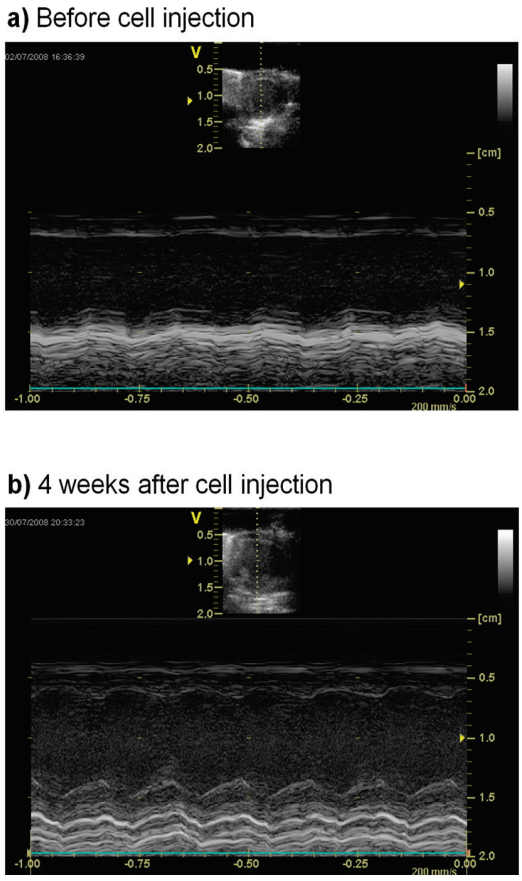
Rats aged 8-11weeks were anesthetized with 1% isoflurane, intubated, and ventilated by a rodent ventilator with 1% isoflurane in a mixture of 1/3 O_2 and 2/3 N_2O. A left thoracotomy was made at the fifth intercostal space, and the proximal portion of the left coronary artery was rapidly ligated by an intramural suture to induce anterior wall myocardial infarction.[72, 73] The chest was closed, subcutaneous Buprenorphine 0.05mg/kg was administered for analgesia, and animals were monitored for one hour after surgery. After 6 days, rats were sedated with 1.2 % Isoflurane inhalation and transthoracic echocardiography was performed. Animals with fractional shortening (FS) <25 % were selected for further studies as this cut-off identified subjects with significant myocardial infarctions[74], thus having a potential to benefit from regenerative therapy. The following day (day 7), animals were randomized to receive SM-MSCs, ADSCs or placebo (cell growth medium without cells). Sedation, intubation and anaesthetizia were performed as described. The chest was the reopened by median sternotomy, the infarct zone was localized, and a total of 150 µL cell suspension or placebo was injected as 4 aliquots (3 in the border zone and one in the infarct zone), with a 30G needle. The chest was then closed and desufflated through an 18 G drain. Postoperative analgesia and monitoring as described above. Four weeks after cell injections, animals were weighed, repeated echocardiographies were performed. Animals were then euthanized, the hearts were harvested for histological analysis and the length of the right tibia was measured.

Echocardiography

Transthoracic echocardiography was performed on sedated animals in supine position with a Vivid 7 scanner and a 14 MHz Linear Array probe (GE Vingmed Ultrasound, Horten Norway). As both parenteral and gaseous anaesthetics induce changes in LV pre- and

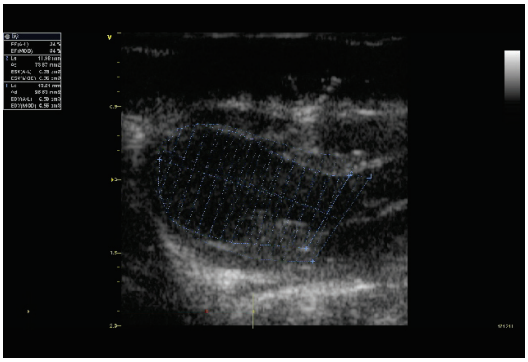
afterload, reduce contractility and even cause LV dilatation, a low dose of isoflurane was used (1.2 %), and rapid image acquisition was pursued. Parasternal long axis and 2 short axis cine loops (midpapillary and basal) were aquired. M-mode registration (LV wall thickness, endocavitary diameter, and FS) was aquired in the midventricular level (figure 2). LVEF was calculated by the area-length method from the long-axis cineloop (figure 3a). We also calculated a wall motion score index in a 13 segment model using the long axis- and two short axis cineloops (figure 3b).[75] All analyses were performed blinded to treatment allocation. Measurements were performed on three consecutive heart-cycles and averaged.

Figure 2. M-mode echocardiograms.



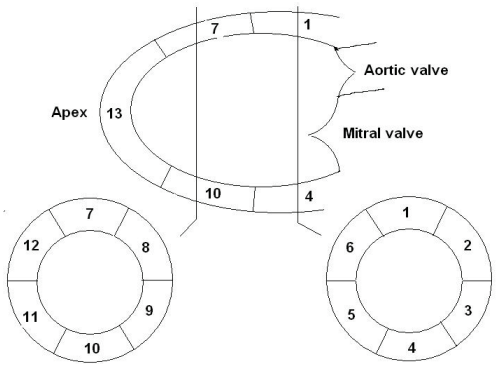
M-mode echocardiograms from one MSC treated animal at baseline and 4 weeks after cell injection. Wall thickening both in the septum and in the posterior wall has improved, but som dilatation of the left ventricle has occurred.

Figure 3a. LVEF by area- length



Parasternal long-axis image with superimposed area-length tracings for calculation of LVEF

Figure 3b.



The 13 segment model of the murine left ventricle, based on parasternal long-axis and two parasternal short-axis cineloops.

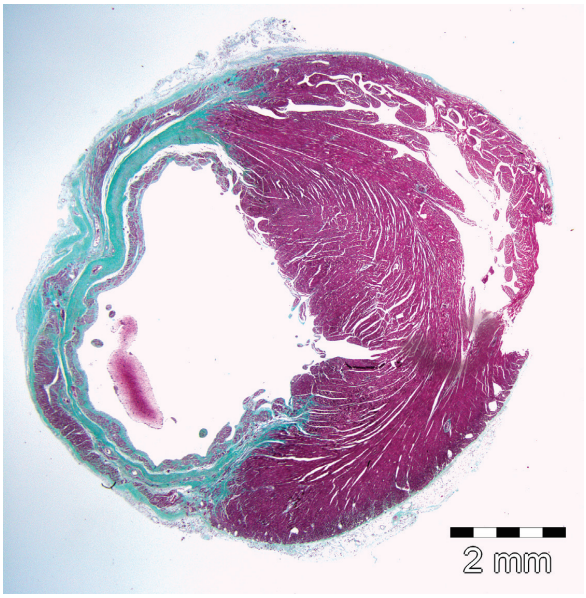
Histological preparation

After explantation, hearts were fixed in phosphate-buffered formalin (4%) for 24 hours, cut in 4 mm slices, dehydrated, and embedded in paraffin. Random hearts in each group were selected for assessment of infarct size and vascular density. Hearts were cross-sectioned in 3 μm sections on a Leica microtome to obtain a stack of short-axis slices covering the entire left ventricle with 1 mm gap between slices for assessment of both infarct size and vascular density.

Infarct size

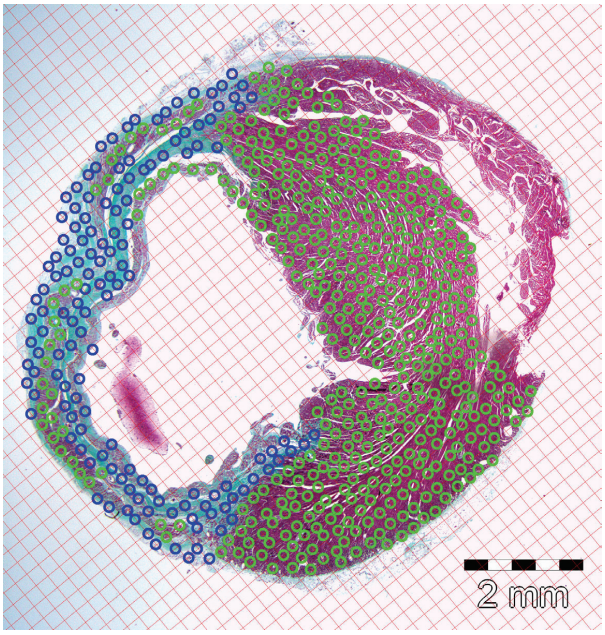
For estimation of infarct size, sections were stained with Masson Goldener (trichrome), according to the standard procedyre in the Department of Pathology, Rikshospitalet. Masson Goldener stains collagen green and viable myocardium stains red, as illustrated in figure 4a. Microscopy was performed by pointcounting images collected at 12.5 x magnification with a randomly superimposed 250 µm x 250 µm grid, and intersections on the grid were recorded as hits on the infarct zone or not, as illustrated in figure 4b. The percentage infarct area on each section was determined by the number of infarct zone intersections on the grid divided by the total number of intersections. The sum of scores for all slides covering the entire LV was used to estimate the total infarct.[76] The entire interventricular septum was counted as a part of the left ventricle. The pericardial zone and the right ventricle was not included in the analysis. An Olympus microscope with AnalySIS software (OlympusSIS, Münster, Germany) was used for morphometry.

Figure 4a.



Cross section of a murine heart stained with Masson Goldener. The green collagen staining indicates a large myocardial infarction. 12.5x magnification.

Figure 4b.

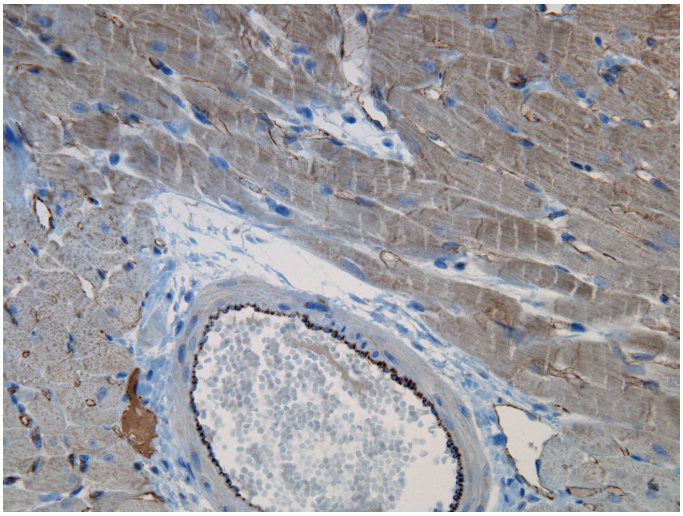


The image in 4a after pointcounting with green markers (non-infarcted left ventricle) and blue markers (infarct zone) on a superimposed grid. 12.5x magnification.

Vascular density

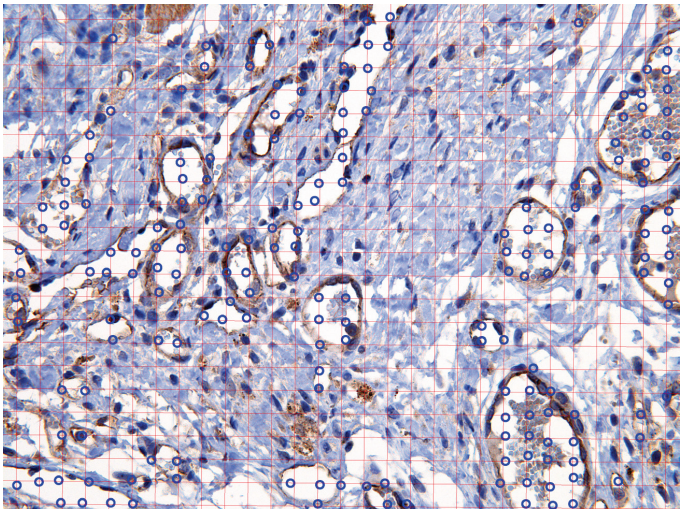
To assess vascular density, sections were stained with goat anti-VE Cadherin primary antibodies (R&D Systems, Minneapolis, MN), biotinylated rabbit anti-goat secondary antibodies (DAKO, Glostrup, Denmark)), and streptavidin peroxidase. Hematoxylin was used for contrast staining (figure 5). Microscopy was performed on images collected with 400 x magnification on a 15 x 15 μm randomly superimposed grid. 2 representative fields in the infarct zone (scar tissue), borderzone (granulation tissue), and remote myocardial segments were counted on 6 representative sections from 10 random animals in each group. The fields were selected according to a prespecified system for localization on the slide, in order to reduce potential bias. The number of intersections affecting vessel lumen and -wall was divided by the total number of intersections in the relevant tissue on the slide to obtain the density of VE Cadherin positive vessels (figure 6). As larger vessels (diameter > 50 μm) were rare events in the myocardium and in the scar tissue, these were not included in the analysis.

Figure 5.



Normal myocardium with anti VE-cadherin and hematoxylin staining for calculation of vascular density. 400x magnification.

Figure 6.



Borderzone with VE-cadherin staining and superimposed grid for calculation of vascular density. Blood vessels marked with blue circles. 400x magnification.

Borderzone quantification

The borderzone was defined as the potential area between the scar tissue and the myocardium. In our study, the borderzone was characterized by the presence of granulation tissue (figure 7a and 7b). Granulation tissue was defined as loose connective tissue, rich in small blood vessels and free from cardiomyocytes or collagenized scar tissue. To quantify the granulation tissue in the borderzone, we obtained one 100 x image from the borderzone on both sides of the infarction (both sides of the infarcted sector of the circumference). Granulation-tissue was quantified by morphometric analysis on a 150x150 µm superimposed grid, pointcounting granulation tissue (hits) and other cardiac tissue (non-hits), as previously described. The pericardial zone and the right ventricle were not included in the analysis.

Figure 7a.

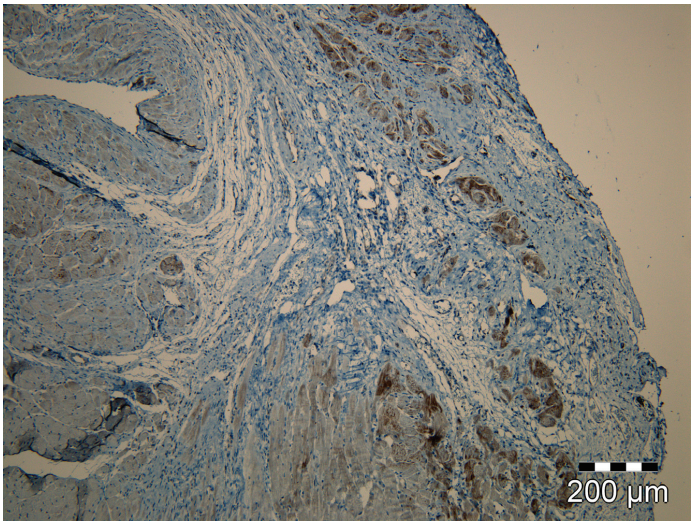


Image from the borderzone in a heart from the MSC group. 100x magnification.

Figure 7b.

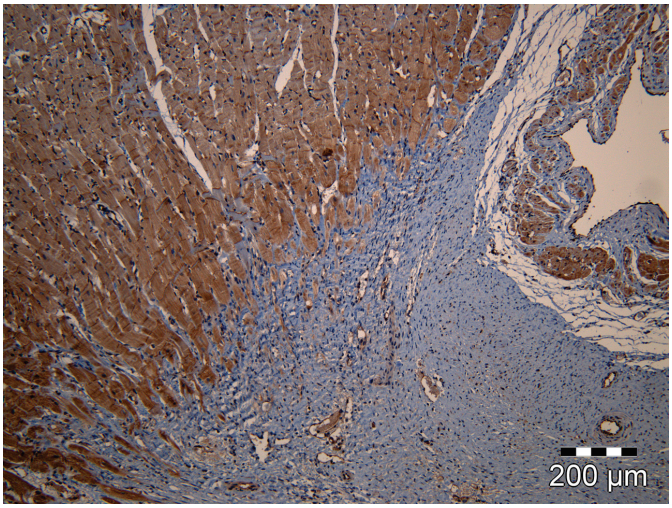
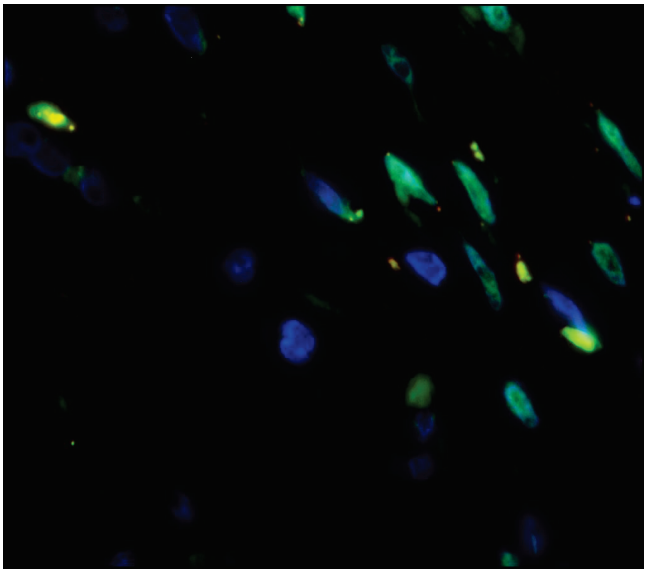


Image from borderzone in a heart from the placebo group. 100x magnification.

In situ hybridization

For identification of the transplanted cells, we performed fluorescence in situ hybridization (FISH) with a fluorescein isothiocyanate (FITC)- conjugated probe specific for the human ALU- sequence (Alu positive control probe, Ventana, Tucson, AZ) on sections incubated in a Ventana discovery machine according to the manufacturer's instructions. For initial cell tracking, the fluorescent ALU- signal was amplified by incubating the slides overnight with Alexa 488 conjugated mouse anti-fluorescin antibody (Millipore, Billerica, MA), as illustrated in figure 8. On slides used for differentiation staining, the ALU-signal was not amplified.

Figure 8.



Infarct zone stained with FITC conjugated ALU-probe (green human DNA) and 4',6-diamidino-2-phenylindole (DAPI) (blue cell nuclei). 600x magnification.

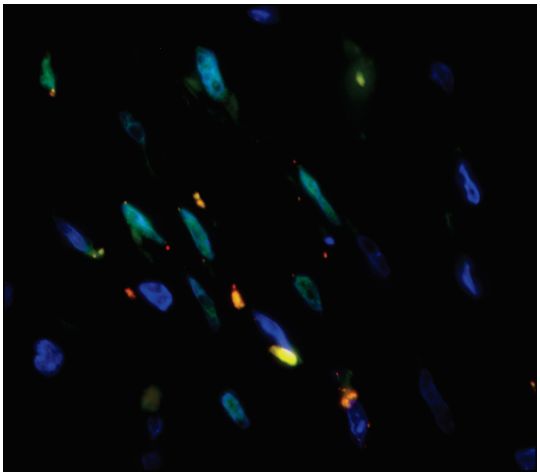
Immunofluorescence staining

Tissue sections with Alu-positive cells were counterstained with myocardium-, smooth muscle-, and endothelium-specific antibodies. Primary mouse anti-human Mabs specific for anti-smooth muscle actin (SMA, Dako), anti-desmin (Dako), anti-CD 31(Dako), and anti-Nkx 2.5 (R&D Systems), and Alexa 555-conjugated anti-mouse secondary antibodies (Molecular Probes, Eugene, OR) were used. We also applied rabbit anti-Troponin I primary antibody (Abcam, Cambridge, UK) with biotinylated goat anti-rabbit (Vector Labs, Burlingame, CA) and Alexa 594-conjugated streptavidin (Molecular Probes). Nuclei were stained with DAPI. Sections from human hearts and hearts from rats in the placebo group were used as positive and negative controls, respectively. Multiplane fluorescence microscopy was performed on a Zeiss Axioplan 2 microscope (Göttingen, Germany) with ISIS software (Metasystems, Altlussheim, Germany). All image analyses were performed blinded regarding treatment allocation.

Figure 9 a-d.

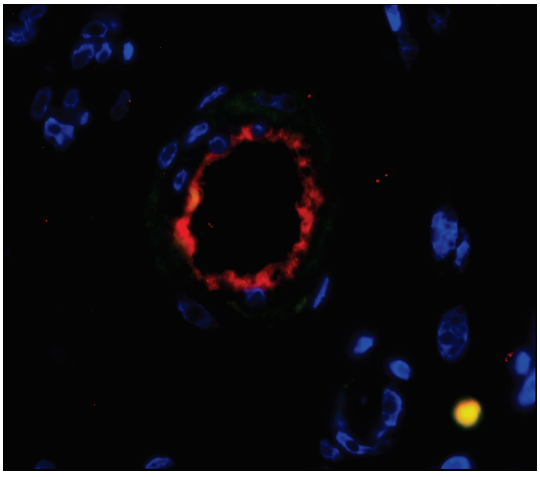
Tissue sections from a heart treated with SM-MSC injections. All sections stained with DAPI (blue nuclei), FITC conjugated Alu-probe (green and blue nuclei). Additional antibodies with red fluorescence against markers of differentiation as described for each image

Figure 9a.



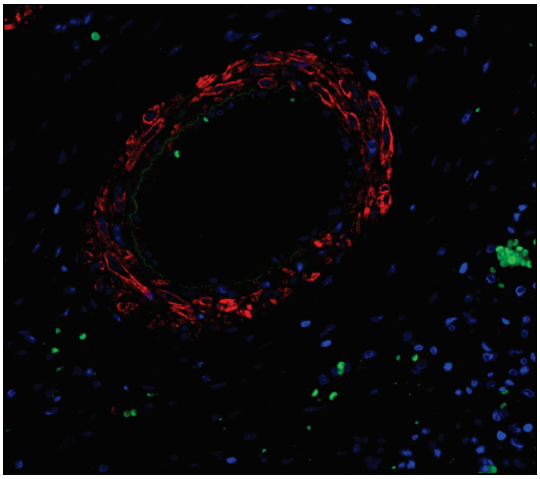
Human troponin I. (Transplanted cells detected, but no human troponin I). 600x magnification.

Figure 9b.



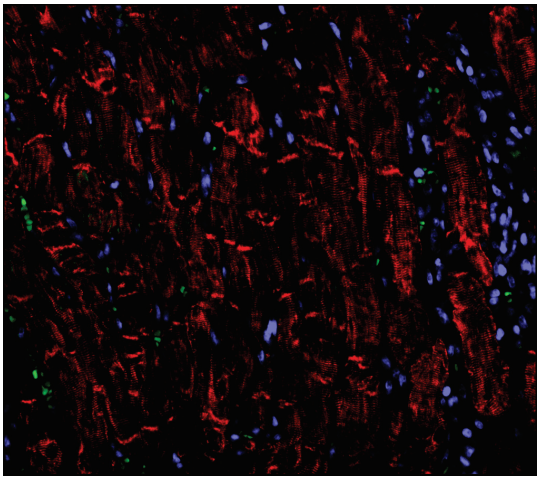
CD 31. (No human cells detected) 600x magnification.

Figure 9c.



SMA. (No human cells identified. The small green signals are RBCs (autofluorescence)). 200x magnification.

Figure 9d.



Desmin. (No human cells identified. The small green extranuclear signals are RBCs (autofluorescence)). 200x magnification.

Statistics

Baseline characteristics were presented as mean±standard deviation (SD) for all animals included in the study. Follow-up characteristics were presented for all animals surviving to follow-up. Change in characteristics was calculated only for animals surviving to follow-up. All continuous data were analysed by one-way ANOVA with Bonferroni correction for multiple comparisons in the post-hoc analyses. Categorical variables were analysed by Chi-square tests. SPSS software version 15.0 and 16.0 was used.

Ethics

Skeletal muscle and adipose tissue were obtained from voluntary human donors after written, informed consent. The collection and storage of samples and cells were approved by the regional committee for ethics in medical research (approval number 1.2006.740 and 2.2007.132, respectively). The use of animals was approved by the National Animal Research Authority, and animals were handled in accordance with the Norwegian Animal Welfare Act and the Norwegian Regulation on Animal Experimentation.

Summary of the results

Paper I: Data from the 3 year follow-up of patients in the ASTAMI study were presented. The rates of adverse clinical events were low, and did not differ between groups. By MRI and echocardiography, no difference was found between groups in LV volumes, LVEF or infarct size at any point of time, and the change over time was also similar. Infarct size measured by MRI decreased significantly in both groups during the 3 years of observation. On exercise testing, we found a larger improvement from baseline to 3 years in exercise time in the mBMC group as compared to the control group. Similar results were found after 6 months. No differences between groups were observed for other parameters on exercise capacity, including the predefined endpoint peak VO_2. On the SF 36 QoL questionnaires, the patients reported perceived physical and mental health status in close range to age-adjusted healthy normal population, and there was no differences between the groups. Blood biochemistry analyses did not reveal significant abnormalities, median pro- BNP level was within the normal range, and there were no significant differences between the groups.

Paper II: Data from a comprehensive analysis of echocardiographic data aquired during 3 years follow-up of the ASTAMI pasients were presented. Transthoracic echocardiography was performed at baseline, 3,6,12 months and 3 years. From baseline to 3 years, LV ejection fraction changed from 45.7 % to 47.5 % in the mBMC group, and from 46.9 % to 46.8 % in the control group (p=0.87 for difference in change over time between groups). Longitudinal strain in the LAD territory improved from -9.7 % to -12.2 % in the mBMC group and from -9.9 % to -12.8 % in the control group (p=0.45). WMSI also decreased significantly in both groups. E/e' decreased from 14.7 to 12.9 in the mBMC group and from 14.8 to 11.9 in the control group (p=0.31). In both groups, there was significant decrease in peak E, increase in peak A and increase in DT, all indicating a decrease in filling pressure during the 3 years follow-up.

The results indicated improvement in both regional and global systolic LV function and diastolic LV function during 3 years follow-up after AMI. No significant differences between groups indicating any effect of mBMC therapy was observed, even when a new and more sensitive methods like 2D-STE was applied.

Paper III: MSCs from skeletal muscle (SM-MSCs) and adipose tissue (ADSCs) were injected in the myocardium of nude rats one week after myocardial infarction. After four weeks of observation, LVEF was significantly improved in the SM-MSCs group (39.1±9.7%), and in the ADSC group (39.6±6.8%), compared to the placebo group (31.0±8.3%, P<0.001 for difference in change between groups). Infarct size was smaller after cell therapy (16.3±4.0% for SM-MSCs, 15.8±4.9% for ADSCs vs 26.0±6.8% for placebo, P<0.001), and the amount of highly vascularized granulation tissue in the borderzone was significantly increased in both groups receiving MSCs (18.3±3.7% for SM-MSCs, 22.6±6.0% for ADSCs vs 13.1±5.3% for placebo, P=0.001). By in situ hybridization, moderate engraftment of transplanted cells was found, predominantly in scar tissue close top the borderzone. No transdifferentiation to cardiomyocytes, endothelial cells, or smooth muscle cells was observed.

Discussion

This thesis demonstrates that intracoronary injection of mBMCs in patients after acute anterior wall myocardial infarctions is feasible and probably safe in a 3 years perspective, but significant beneficial long term effects on predefined endpoints like LVEF, infarct size, peak oxygen consumption, and quality of life were not found. In the experimental study using a xenomodel with human cells transplanted into nude rats, we found that MSCs harvested from both adipose tissue and skeletal muscle were able to engraft in the heart and significantly improve LVEF when the cells were injected intramyocardially one week after myocardial infarction. Infarct size was reduced, but the injected cells did not differentiate to cardiomyocytes.

Methodological considerations, clinical study

The present study was a prolonged follow-up of patients in the ASTAMI study. As the long-term follow up was not part of the original protocol, the results should be considered exploratory. We have used the same methods for data collection at 3 years as in the original protocol, to be able to perform analyses of longitudinal changes in addition to cross-sectional comparisons between groups. We also used the same parameters as primary endpoints to reduce the risk of type I errors by multiple testing. ASTAMI was a phase II study evaluating efficacy, safety and feasibility of intracoronary mBMC injections in acute AMI. The sample size was inadequate for efficacy testing on clinical endpoints, and change in LVEF was used as the primary surrogate endpoint. For exercise testing, change in peak MVO_2 was selected as the predefined endpoint. Several issues regarding the original protocol have been discussed in previous publications.[24-26, 28] In the current discussion, issues unique for or most relevant for the long term follow-up are emphasized.

Internal validity: To avoid *selection bias*, the ASTAMI-study was designed as a randomized study, and all analyses in the study were performed according to treatment allocation (*intention to treat)*. At baseline, no significant differences between groups were found. In the linear regression model applied for analysis of longitudinal change in parameters with multiple (>2) observations, adjustment for baseline value was included in the model to minimize eventual impact of small (random) differences between groups at baseline. For the same reason, ANCOVA was used for comparison between groups when paired data were available. One patient originally included in the study and randomized to

the control group experienced an early myocardial reinfarction with cardiogenic shock. The patient underwent cardiac transplantation and was excluded from the study at day 11, as data on the primary endpoint, change in LV- function, were no longer available. Another patient in the control group was not willing to participate in the 3 years follow-up. The patient did not withdraw consent for the original study. The patient has been registered as alive at 3 years, but no further data are registered at 3 years and values from the 12 months follow-up have not been carried forward. The exclusion of a patient with early reinfarction contribute to the low rate of clinical events in ASTAMI, but the loss of 2 (potential) clinical events from the control group would not significantly alter the conclusion in the study (data not shown).

Regarding *information bias*, ASTAMI was an open labeled study. Sham bone marrow-harvesting and cell-infusion was not performed. Thus, all patients and some of the investigators were aware of the treatment allocation. This may potentially have influenced the registration of clinical events, the patients reporting of quality of life in the SF-36 questionnaire, and also the performance during cardiopulmonary exercise testing. Objective parameters like peak heart rate and respiratory exchange ratio (RER) did not indicate significant difference in efforts between groups during exercise, but we did find a significantly better improvement in exercise time and heart rate recovery in mBMC treated patients despite no difference between groups in the increase of peak VO_2. A placebo effect, or information bias, may possibly explain this observation, as exercise time, but not peak VO_2 or maximum heart rate, can be improved by extended efforts at maximum exercise. Peak VO_2, and not exercise time, was selected as the primary measure for exercise capacity in ASTAMI. According to the considerations on bias, prognostic value, preselected endpoints and the risk of type I errors due to multiple testing, the difference between groups in change in exercise time was not emphasized. In general, at the 3 years follow-up, parameters at obvious risk for subjective influence were similar between groups and corresponded with data from more objective measures. After publication of the results from the 6 months follow-up, patients were aware of the neutral treatment effect. Thus, neither patient information nor collected data suggest significant information bias. All analyses of echocardiograms, MRI, SPECT, laboratory specimens, SF-36 questionnaires, and results from cardiopulmonary exercise testing were performed by observers blinded to treatment allocation and blinded to results from other tests.

Performance of measurements:

-*Clinical endpoints:* In the ASTAMI study, clinical endpoints were registered to monitor the safety of this novel treatment modality. As tabulated in paper I, the rate of clinical events was low in ASTAMI, and there was no significant difference between groups. Patients were monitored regularly, and all clinical events were registered in the case report form (CRF). Only one patient, in the control group, dropped out of the study. Injected mBMCs were not labeled, and patients were not tested for systemic embolization of mBMCs. Patients were not scanned for silent infarctions in common sites for cardiac embolization (brain, kidney, spleen, liver). However, no patient had clinical symptoms or changes in biochemical markers indicating extracardiac embolic or ischemic events related to cell therapy. Procedure related myocardial infarction or subsequent reinfarctions were diagnosed according to current guidelines.[77] Other clinical events were registered based on routine evaluation by the investigator. All adverse events were reported to the independent data safety and monitoring board (DSMB) for evaluation. With an open-label design, awareness of treatment group may potentially influence the reporting of clinical events. However, despite low event-rates, we have no indications for biased reporting of adverse events in ASTAMI. In a study with 100 included patients and low rate of clinical events, it is important to underscore that there is not sufficient statistical power to evaluate treatment efficacy on clinical endpoints, implicating that we can neither reliably conclude that the treatment is safe, nor rule out a potential clinical benefit of mBMC therapy.

Left ventricular function: The primary endpoint in ASTAMI was the change in LVEF from baseline to 6 months by SPECT. At the 3 years follow-up, we evaluated the risk-benefit relation for every examination in the original protocol, and did not perform coronary angiography or radionuclide scans.

Cardiac MRI was performed at 3 years, and provided supplementary data regarding infarct size and safety, as tumor formation and calcification has been reported as potential side effects after mBMC therapy. Regarding LVEF by MRI, a complete dataset after 3 years was available in 84 patients. Thus, 13 patients had contraindications or missed the baseline recording due to logistic problems. The baseline MRI scans were performed 2 weeks after mBMC injections. Thus, eventual effects of mBMC therapy appearing earlier than 2 weeks after injection would not be detected and reflected in the MRI data. The late enhancement imaging was cancelled in one patient at 3 years due to reduced renal function, as the potential risk for nefrogenic systemic sclerosis with use of Gadolinium contrast agents had

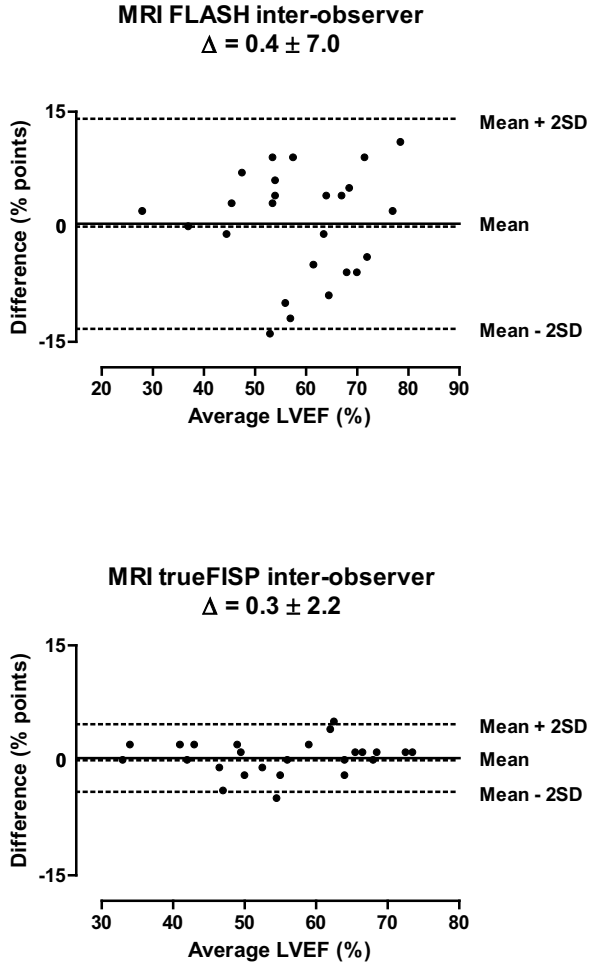
been further acknowledged at this time. The MRI dataset was also limited by the use of old MRI scanners (Magnetom Vision Plus) and the Compact-FLASH (n=63) processing protocol in the first included patients. These images had poorer image quality, resulting in higher interobserver variability compared to analyses performed with new equipment and new processing protocols (Magnetom Sonata and True FISP), as illustrated in figure 10. Furthermore, LVEF has been calculated by the biplane area length method on 2-chamber and 4-chamber cine images. The current gold standard for calculation of LVEF is the Simpson method based on a stack of multiple short-axis cine images covering the entire left ventricle. Although a comparison of the 2 methods published before ASTAMI indicated high correlation[56], the biplane method carry risk of foreshortening compared to the true length axis of the ventricle. The image planes may also vary between different examinations in the same patient, leading to variability especially in patients with regional myocardial dysfunction. At the 3 year follow-up, both short-axis and long-axis cine-images were acquired, and data for comparison are presented in table 2.

Table 2.

	Short-axis method (mean± SD)	Area-length method (mean± SD)	Difference (mean± SD)	Intraclass correlation
EDV (mL)	175±63	164±61	12±17	0.96
ESV (mL)	86±54	78±48	7±13	0.98
LVEF (%)	54±13	55±12	-0.6±6.3	0.87

Comparison of the area length method and the multiple short-axis Simpsons method for calculation of LV volumes and LVEF by MRI.

Figure 10.



Bland-Altman plots for MRI interobserver variability with Compact FLASH and True FISP processing protocols. Illustration from the thesis "Treatment of acute myocardial infarction with intracoronary administration of autologous bone marrow cells". Ketil Lunde. University of Oslo, 2007. ISBN 978-82-8072-762-6. Reused with permission.

As illustrated, the correlation between the 2 methods is excellent for LV volumes, and good for LVEF. Both EDV and ESV are systematically larger when calculated by the multiple short-axis method, and the main reason is probably foreshortening when the area-length biplane method is applied. It is important to acknowledge that although LVEF by the two methods correlate by less than 0.9, the mean estimates by the two methods for LVEF in the patient cohort differ by only 0.6 %. A Bland-Altman plot (not shown) indicates symmetric variance along the entire spectrum of ejection fractions measured in the study. This

indicates that the use of the area-length method for calculation of LVEF in ASTAMI did not introduce drift or bias as compared to the gold standard multiple short-axis method.
MRI is considered more precise than echocardiography in test-retest evaluation of LVEF.[78-80] Due to limitations regarding the timing of the first MRI recording, the MRI data in our population may not accurately reflect the effects of mBMC therapy from baseline, but should be considered more precise than echocardiography in evaluation of changes in LV volumes, LVEF, and infarct size between 2 weeks and 6 months, and between 6 months and 3 years.

Transthoracic echocardiography was performed at baseline, 3,6,12 and 36 months. The baseline recording was performed 4.5±1.1 days after AMI, i.e. 1-2 days before cell injection. As the first MRI scans were performed approximately 2 weeks after cell injection, the change in LVEF by echocardiography remains the most reliable endpoint for effects of mBMC therapy on LV function in our 3 years follow-up. 2D-STE analyses were performed by Ola Gjesdal (OG, n=49) and Jan Otto Beitnes (JOB, n=51). 2D-STE analyses were performed by the same observer at all timepoints to eliminate interobserver variability in change in strain over time. All other echo-derived data are based on analyses performed by Ketil Lunde (KL) at baseline, 3, 6, and 12 months, while JOB performed the analyses at 3 years. As the same observer analysed recordings from both groups at each point of time, while the observer was replaced between 12 months and 3 years, evaluation of interobserver variability was emphasized. Interobserver variability between KL and JOB was tested by JOB reanalyzing a random sample of 25 echocardiograms from the 6 months follow-up. Results are presented in table 3 and figure 14 a-d. Interobserver variability on global strain between OG and JOB was tested by JOB reanalyzing a random sample of 25 echocardiograms from the 6 months follow-up. The difference between observers was 0.75±0.22 %.

Table 3. Interobserver variability. Standard echocardiography.

	KL (mean± SD)	JOB (mean± SD)	Difference (mean± SD)	Intraclass correlation
EDV (mL)	149±49	147±45	-1±14	0.96
ESV (mL)	79±31	77±32	-1±8	0.97
LVEF (%)	48±10	49±8	1.1±4.9	0.85
WMSI	1.58±0.21	1.61±0.25	0.04±0.13	0.83

These interobserver analyses confirm that variability between observers was acceptable. Bias was only 1 mL for LV volumes and 1 % for LVEF. Standard deviations were also low enough to assume that level of statistical power was maintained.

External validity

The result in ASTAMI differs from REPAIR-AMI, where the event rate was higher and there was a significant reduction in major adverse cardiovascular events (MACE) in the mBMC group after 1 year, as compared to placebo. The mortality in ASTAMI also differs from clinical registry data, i.e. 30 day mortality after ST-elevation AMI was 11.1 % in the European Heart Survey (EHS) in 2004.[81] Most clinical trials will recruit patients with low comorbidity and high compliance with the study protocol and other advice, to reduce noise in the dataset. Thus, the risk of adverse clinical events will often be lower than observed in the registries. This selection of patients may reduce the external validity of the study, i.e. whether the results from the study can be extended and applied in a general patient population.

Table 4 illustrate selected characteristics of patients in ASTAMI compared to patients from other studies; ASTAMI and REPAIR-AMI are mBMC studies, HORIZONS- AMI[82] is a contemporary study on patients with acute STEMI comparing different types of stents and antithrombotic therapy in the cath- lab, Europeans heart survey (EHS 2004) is an European registry from selected hospitals.

The data in Table 4 can, to some extent, clarify why mortality in ASTAMI was low despite large anterior wall ST- elevation myocardial infarctions. In ASTAMI, 1608 patients admitted to RH and UUS in the study period with ST-elevation myocardial infarctions were screened for participation. 718 patients were excluded due to RCA or CX related infarctions, 646 did not match inclusion/exclusion criteria (comorbidities etc), 50 patients were reluctant to participate, and 93 were ineligible due to logistical considerations. Patients in ASTAMI were younger and had lower prevalence of risk factors than patients in the EHS. It should also be noticed that the medical therapy in ASTAMI adhered absolutely to current guidelines, while compliance was slightly lower in both REPAIR, HORIZONS-AMI and in the EHS. In addition, two patients in ASTAMI were successfully resuscitated after in-hospital cardiac arrest during protocolled extended hospitalization (day 7-10).

The table also illustrates that patients in the clinical studies represent selected subgroups in comparison to the general AMI population. In the EHS, only 70 % of patients presenting with ST- elevation AMI underwent coronary angiography. Patients in the mBMC studies

were > 5 years younger and had fewer risk factors and lower comorbidity as compared to patients in the EHS. Characteristics of the 1608 patients screened for ASTAMI were also different from the registry (data not shown). The majority of patients screened at UUS and RH were referred from other hospitals. This may indicate that patients transferred to UUS and RH for acute PCI had already been subject to some selection. Thus, the results in aforementioned clinical trials can probably be extended to most patients with STEMI referred to acute PCI, but not to all patients presenting with AMI.

Table 4

	ASTAMI	REPAIR-AMI	HORIZONS-AMI	EHS 2004
Age (y)	57.4 ± 9.0	56 ±11	59.6	62.5 ± 13.1
Women (%)	16.0	18.0	23.5	25.9
History of previous CABG (%)	0	na[#]	2.1	8.9
History of previous PCI (%)	9	na	8.6	
Previous AMI (%)	4[#]	na[#]	10.0	15.7
Hypertension (%)	34.0	57.0	51.6	50.0
Renal failure (%)	0.0	0.0	15.5	3.8
Diabetes mellitus (%)	9.0	17.0	15.7	21.4
Anterior ST- elevation myocardial infarction (%)	100.0	70.0	41.0	na
Baseline LV function (%)	mean LVEF 46.3	mean LVEF 47.1	median LVEF 50	na
Mortality (%)	0 (6 months)	3.9 (4months)	3.5 (1 year)	7.2 (30 days)
Aspirin (%)	100.0*	97*	98*	97*
Betablocker (%)	100*	94*	92*	83*
Statin (%)	98*	93*	96*	81*
ACE-inhibitor/ARB (%)	100*	97*	83*	75*

†mediaction at 6 months in ASTAMI, 4 months in REPAIR-AMI, at discharge in HORIZONS-AMI and in-hospital in EHS. na= not available

Comprehensive descriptions of prevalence and in-/exclusion of previous AMI, PCI and CABG are not specified in the publications or protocols. In ASTAMI, patients with previous Q-wave infarctions were excluded, and previous PCIs and non-Q infarcts were registered. Such data from Repair-AMI were not published, but it can be presumed from objective and design of these studies that the prevalence of these conditions was low.

Comparison with other mBMC studies

The majority of studies investigating the effects of mBMC therapy in AMI have included 20 to 200 patients, followed the patients for 4-6 months and used change in LVEF as the primary endpoint. Clinical events have been registered for evaluation of safety, as sample size has been inadequate to conclude about effects on clinical endpoints. As seen in table 1, the BOOST and REPAIR-AMI studies demonstrated a statistically significant improvement in LVEF 4-6 months after mBMC- therapy. The other major trials did not provide positive results on the primary endpoint, but there is an overall trend suggesting larger improvement in LVEF in patients treated with mBMCs. This is also reflected in the metaanalysis by Martin-Rendon et al, where an average treatment-effect of 2.99% on LVEF was found after mBMC- therapy.[34] However, it should be noted that the large REGENT[83]and HEBE[84] studies, which both failed to prove significant benefit of mBMCs, were published after this metaanalysis and are therefore not part of the data basis. Furthermore, at least 2 other studies on mBMC treatment in AMI have been prematurely terminated based on negative preliminary results.[37, 85] Only few studies have presented data from follow-up beyond 12 months. In the initially positive BOOST trial, no positive effect of mBMC therapy on LVEF could be detected after 5 years, and the rates of clinical events were similar in the two groups.[86] From the REPAIR- AMI study, where single-plane angiocardiography was used to evaluate LV function at baseline and after 4 months, extended follow-up of LV- function has only been presented from a subgroup of 54 patients included in a substudy with cardiac MRI at baseline, 4 months and 12 months.[87] In this substudy, the groups were small, and although the numeric results corresponded well with findings by angiography at 4 months, there was no significant difference in treatment effect between groups. Thus, the neutral result after 3 years in ASTAMI is not in conflict with long term follow-up data from other studies.

The neutral result in ASTAMI and the positive result in REPAIR-AMI after 4 months have, by some, been regarded conflicting results. Thorough investigations were initiated to identify explanatory differences in protocols and methods. The underlying assumption has been that the cell product in ASTAMI was impaired as compared to cells used in REPAIR-AMI. One study suggested that cell separation by Lymphoprep yielded fewer cells than centrifugation with Ficoll-Paque (Cambrex), and that storage of mBMCs in Heparin-plasma lead to reduced migratory capacity against SDF-1 gradient in vitro, as compared to storage in X-VIVO 10 (Cambrex).[30] However, this study was conducted by members of the

REPAIR-AMI study group, and has to date not been reproduced by others. Another study, by members in the HEBE group, found similar yield of cells with the use of Lymphoprep and Ficoll paque, and suggested that low sentrifugation speed and loss of cells in the supernatant could explain a low yield of cells in ASTAMI.[88] However, this study did not use the actual ASTAMI centrifugation protocol, and in ASTAMI it was checked that the supernatant did not contain cells. It has also been suggested that red blood cells (RBCs) in the cell product will reduce its clinical efficacy.[89] This has not been prospectively addressed in any clinical study, and the gelatine-polysuccinate density gradient sedimentation method applied to isolate BMCs in the initially positive BOOST-study, yield a cell product with a relatively high content of polynucleated white blood cells (WBCs) and also quiet a few RBCs. Thus, details in cell preparation may be of importance, but the findings in these post-hoc studies remains controversial and should be prospectively evaluated in clinical trials. With time, several larger studies with well characterized cell products have failed to prove significant effects of mBMC therapy. Meta- analyses have found overall treatment-effects around 3 % on LVEF.[34] In REPAIR-AMI, the treatment effect was 2.5 %. ASTAMI was designed with 80 % power to detect a treatment effect of 5% on LVEF by SPECT. Thus ASTAMI was not powered to detect the modest beneficial effects on LVEF found in some other trials, and is as such not in conflict with other evidence. Furthermore, the beneficial effect of mBMC therapy seems limited as most trials fail to meet their primary endpoint.

To summarize, small to medium sized clinical trials suggest a treatment effect on LVEF within the range 0-3 % when autologous mBMCs are harvested and injected approximately 1 week after AMI. The procedure is considered safe, based on summarized clinical endpoints in the meta-analysis.[34] Whether the modest effect on LVEF eventually translates into a significant reduction in mortality, is not clear. In the REPAIR-AMI study, a significant improvement in clinical outcome was observed after 1 year.[90] This was not the primary endpoint, and the result was mainly caused by a high rate of coronary events in the placebo group, as discussed elsewhere.[33] A pan-European initiative to run a prospective randomized trial with 3000 patients and statistical power to address effects on clinical endpoints is in progress (The BAMI trial, prof. A. Mathur, personal communication).

Methodological considerations, experimental study

LV function: We used transthoracic echocardiography for evaluation of LV function. Image quality allowed tracing of endocardial contours and wall motion scoring in all animals.

In our department, LV fractional shortening (FS) has been the most frequently used parameter for evaluation of LV function in heart failure studies in rats. Time resolution with M- mode imaging is high, which is advantageous when heart rate (HR) is high. However, FS is measured at the midventricular level (tip of the papillary muscle) and the posterolateral segment will not be infarcted after LAD- ligation while the mid-septal segment will be variably affected depending on coronary anatomy in different animals. Thus, the measurements of FS may have been performed in infarct zone, border zone or normal myocardium, depending on infarct size and localization after LAD- ligation. FS measurements reflect changes of LV-dimensions along one tangential line in a single plane. This method can be fairly accurate for conditions with uniform influence on the myocardium, while conditions with regional changes in LV function can be misjudged. According to the limitations of the FS- method, and the regional nature of both the ischemic lesion and the stem cell injections, we also measures LV systolic function by area-length derived LVEF and wall motion scoring.

The area- length-method for estimation of LVEF has been used for over 50 years and is based on tracing of endocardial contours and estimation of the LV length axis on 2D images at the end of diastole and the end of systole.[91, 92] In our study, parasternal long-axis images were used. ECG was not registered, and frames with the smallest and largest areas during the heart cycle were used. A representative image with trace is presented on page 28, and the formula for calculation of volume by area-length is presented on page 19. Although this method also estimated LV–function by analysis in a single plane, both radial and longitudinal dimensional changes in segments including the infarct zone were invariably included in the measurement. The other main limitation is framerate. The obtained framerates on 2 D images were in the range 105-200 s^{-1}, depending on the size of the sector, and HR was 394±42 min^{-1} during baseline recordings and 392±35 min^{-1} after 4 weeks. Thus, approximately 15-35 frames/cycle were obtained. As dV/dt is close to 0 at the end of diastole- and - systole, framerates at this level will introduce only modest variability in calculation of LVEF. At the baseline study, FS and LVEF correlated with Pearson R 0.63, $p<0.001$.

Wall motion scoring is a semiquantitative visual estimation of change in wall thickness. In humans, the method is accurate for estimation of LV function and prognisis, especially in regional processes like AMI.[93] In the rat, apical images are not easily obtained without foreshortening. Morgan et al has validated a 13 segment model for WMSI in rat.[75] We slightly modified this model (rotation of the segments in short axis view for better

correspondence between short-axis and long axis segments), and applied it for estimation of a WMSI in our study. The main limitation was reduced visualization of medial and apical segments in some rats. Wall motion scoring may also lack sensitivity for changes in the degree of hypokinesia, and it may also be difficult to reflect changes in the extent of akinesia within a segment, as the scar volume contracts over time.

For all parameters on LV function measured during general anesthesia or sedation, cardiodepressive pharmacological effects will play a role. Inhalation of isoflurane provides rapid onset, easy adjustment and immediate recovery of the sedative effect. With short induction and a low dose of gas for inhalation, hypothermia and cardiorespiratory depression could be minimized. We sought to perform a standardized procedure for sedation and echocardiographic recordings to minimize variation in depth and length of the sedation. During pilot studies, we observed significant LV dilatation, decreased contractions and bradycardia with higher gas concentrations and/or prolonged sedation, especially in the presence of large MIs. The cardiodepressive effect is reflected in heart rate. The relatively high heart rates registered during our echocadiographic recordings, and the similar heart rates at the initial and the final examination, correspond well with a standardized procedure with low gas concentrations.

Infarct size

Estimation of infarct size in tissue post-mortem can be performed with a number of enzymatic and immunohistochemical stainings. In the early phase after coronary occlusion, it is difficult to distinguish reversible from irreversible damage. In contemporary experimental models, the triphenyl tetrazolium chloride (TTC) method is frequently used. TTC stains viable tissue red by a dehydrogenase dependent reaction, while the infarct zone is dehydrogenase deficient and remains unstained.[94] It can be used early, ideally after 3 hours of reperfusion to allow washout of dehydrogenase from the infarct zone, and is usually applied on 2 mm slices of fresh tissue after a freeze- thaw cycle. In acute models, scar formation and remodeling reduce the relative infarct size from 3 days and further, reducing the accuracy of TTC.

When a collagenized scar has developed (after 2-3 weeks), histological staining for collagenized scar tissue, like Sirius red or trichrome staining, is frequently used. Indeed, Masson trichrome stained tissue sections have served as a gold standard for infarct size.[95] As the scar tissue will contract over time, this method will report a smaller infarct size, both absolute and relative, as compared to TTC staining or other measurements performed early.

In our study, we did not use a reperfusion model, and hearts were harvested 4 weeks after the AMI. Furthermore, we were interested not only in infarct size, but also cell engraftment, differentiation, morphology and vascularization. The relative infarct size in comparison between groups was of interest, and similar time had elapsed since the AMI in all groups. Thus, collagen staining was appropriate to meet our needs, and staff with experience in Masson Goldener staining was available in the Department of Pathology.

The method for quantitative measurement of infarct size was based on the following considerations:

First, since animals had variable weight due to the use of both genders, some variation in age and variable presence of oedemas and cardiac cachexia, a relative measure for infarct size should be used, i.e scar tissue as a fraction of heart tissue. As paraffine embedded tissue was available, and tissue sections were also needed for cell tracking and other analyses, scar tissue throughout the heart would best be evaluated by the use of full volume stack of serial short axis tissue sections. The amount of infarct or scar on each slide can be quantified in several ways. Infarct zone has been measured as sector of a 360 degree circumference. Others have used graphic software to delineate infarcted areas. However the presence of variable transmurality, scattering, and islets of regenerative cells limit the applicability of the sector method, and the delineation method may also be difficult to apply if there are cracks in the tissue on the slide, scattering of fibrotic tissue, or islets of regenerated or spared myocardium. Software providing the correct size of traced areas on microscopy derived images is important for reliability. In small animals with thin walls even in the left ventricle, a subendocardial rim of myocardium is often spared after coronary occlusion, resulting in high variation in transmurality. As cell therapy is expected to be most effective in the border zone, infarct size should be measured in all dimensions. Based on these considerations, we decided to quantify scar tissue by the tissue morphometry approach as described. The size of the grid was determined after a pilot study to ensure adequate numbers of "hits" pr. slide. In the pilot study, we also evaluated infarct size in the groups to estimate the sample size. The grid was superimposed by the computer in a random direction, to exclude bias by a specific orientation of the images. Typically, 600-800 hits or non-hits were counted pr. slide. This method provides high spatial accuracy to overcome the limitations of other approaches, and a similar approach as been applied by others.[96] The main limitation of pointcounting is the workload, but to some degree high methodologic accuracy will allow reduction in sample size.

Cell tracking

If beneficial functional or structural effects after cell therapy are observed, it is of great mechanistical interest to clarify the presence of engraftment of transplanted cells in the recipient. In order to identify even low numbers of engrafted cells, the method should be sensitive. It also had to be applicable in formalin fixed, paraffine embedded tissue, and it had to allow further staining procedures on the same slide for clarification of cell phenotype. In our view, high specificity was also of utmost importance, as transdifferentiation events should not be overreported.

MSCs are not distinguished from other cell lines by a specific phenotypic marker, but rather from characteristics in culture (plastic adherence and multilineage differentiation). MSCs are also present in the recipient animals. Thus the donor cells had to be identified either by staining all cells with a fluorescent marker before transplantation or by identification of donor-derived cells in situ with another specific marker- for instance a marker specific for human cells. Other studies have used transgenic cells expressing fluorescent proteins like green fluorescent protein (GFP). GFP may affect functionality of donor derived cells[97, 98]. Functional adverse effects of other transgenic proteins have been even less investigated. Fluorescent substances like Dil or PKH 56 dissolves in the cell membranes of donor cells and provide reliable fluorescent signals before transplantation. However, evidence suggest retention of only a small percentage of cells at the site of injection.[52] The proportion of cells washed out vs. the proportion of cells dying in situ, either by ischemia, apoptosis or destruction due to non-specific or specific immune responses has not been clarified, and soluble fluorescent markers may cause background signals from membrane fragments without relation to viable cells after cell destruction in situ. In case of cell proliferation, we would also expect attenuation of fluorescence from a dissolved marker in the mother cell membrane.

After transplantation, human donor cells can be distinguished from murine recipient cells either by immunostaining against human specific epitopes (proteins) or by in situ hybridization to human specific DNA (or RNA) sequences. Antibodies specific for human HLA-ABC have been used, but the assay has only been successful in frozen sections. FISH have been used for cell tracking either by using a FITC- conjugated probe against the human specific ALU- sequence[99] or by using probes specific for the X and/or Y- chromosome.[100] The signals from the X and Y chromosomes are very small, appearing as a small spot within the nucleus. The size of the signal could affect sensitivity and feasibility during screening for transplanted cells. Also, information about gender of our donors was not available. To

allow co-staining for markers of cell phenotype, it was appropriate to use only one bandwidth for cell tracking. Based on these considerations, we decided to use the Alu probe. Pilot studies were performed to ensure that the FISH procedure did not interfere with the result and specificity of the subsequent immunostaining, and that immunostaining did not attenuate the FITC- signal. During these studies, we did not identify background fluorescence in the cell nuclei that could lead to misinterpretation of the FITC signals. There was moderate autofluorescence from cross-striated muscle like the myocardium, but this appeared mainly in the red spectrum and was confined to the cytoplasm. RBCs and eosinophilic WBCs were also autofluorescent, but these signals were easily identified by size (RBCs are small), localization (RBCs were mainly clustered within blood vessels), morphology (RBCs were not nucleated, eosinophils had bilobed nuclei) and the bandwith of the signal (autofluorescence is oftenly not specific to one band).

Staining for phenotypic markers

The antibodies for staining to clarify differentiation of the injected cells were selected according to the following priorities:

- They had to perform with an acceptable signal to noise ratio in formalin fixed paraffin embedded tissue slides without interfering with the FISH signal.
- If available, human specific antibodies were used, to ensure signals specific for donor derived cells.
- Minimal background fluorescence.
- Availability and former experience with the antibody in our department. A selection of antibodies to cover the most relevant cell types expected to be involved in myocardial regeneration was pursued.

Troponin I is a structural protein located in the cytoplasm of mature cardiomyocytes. It is highly specific for cardiac tissue.

Nkx 2,5 is a protein appearing in immature cardiomyocytes/ cardiac precursor cells. It was used mainly to ensure that we did not overlook transdifferentiated cells too immature to express troponin.

Desmin is a structural protein involved in the cell-cell binding and interaction (the intercalated disk). Its presence in donor derived cells would indicate the formation of gap

junctions- a prerequisite for rapid intercellular conduction of action potentials during cardiac depolarization.

α Smooth Muscle Actin (SMA) is a structural protein specific for smooth muscle, typically found in the walls of small and medium sized arteries.

CD 31 is a marker specific for endothelial cells.

Red fluorescence conjugated secondary antibodies, and for anti-troponin I, biotinylated secondary antibody with a red fluorescence conjugated streptavidin tertiary antibody, were selected.

All antibodies were tested for sensitivity and specificity in human hearts and rat hearts with positive and negative control stainings. After FISH and the subsequent co-stainings, slides were mounted with Vectashield with DAPI. The final staining protocols performed well to obtain sections with blue fluorescent nuclei (DAPI), green fluorescent FITC signals in nuclei in human cells, and red fluorescent signal from the differentiation-staining.

Vascular density

For evaluation of vascular density, we performed enzyme- linked immunostaining against blood vessels in rats. Only few antibodies perform well in paraffine embedded rat heart tissue. Despite some background staining in the myocardium, we selected the VE- cadherine antibody after extensive testing and titration. VE- cadherin (Cadherin-5) is an endothelial specific molecule localized in the adhesion between endothelial cells.[101] The analyses for vascular density required a high number of slides, and visualization of tissue morphology was important. Therefore, light microscopy was preferable. Diaminobenzidine (DAB)/ peroxidase was used as the chromogene assay, and haematoxylin was used as counterstaining for better morphology. Vascular density can be evaluated in several ways, and there is no generally accepted standard for analysis. Most publications describe either the number or the area of vessels stained with a given marker within a “representative” area of tissue. After reviewing the litterature and performing microscopy of some tissue slides, it was apparent that the size of blood vessels was highly variable, the vascular density- measured either by number or by area- was very different in different tissues (myocardium, scar, other stromal/ connective tissue) and the composition of tissue was different on different sections, depending on the presence and the size of an infarct zone and a border zone. Based on these considerations, we decided to vascular densities separately and specific for scar tissue, borderzone connective tissue, and the intact myocardium. The field

selected for quantification was determined by a prespecified pattern for localization, as illustrated in figure 11.

Figure 11.

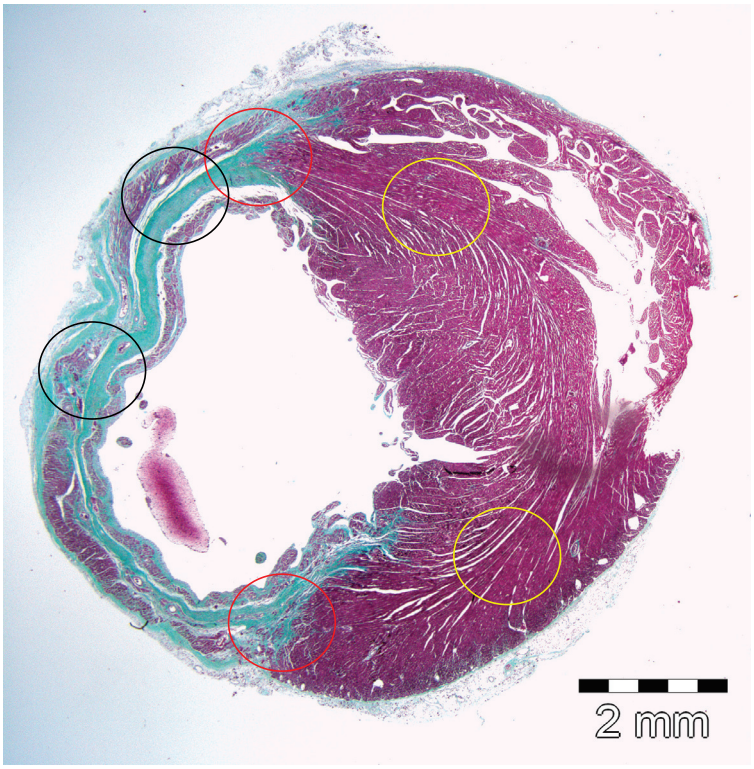


Illustration of the prespecified pattern for selection of microscopy-fields for determination of vascular density. The circles, representing the fields, are superimposed on a Masson- Goldener stained tissue section. Black circles= scar tissue. Red circles= borderzone. Yellow circles= remote myocardium. 12.5x magnification

On each section, we counted two separate fields from the scar tissue. We aimed for two fields with high transmurality as far apart as possible. High transmurality had priority to obtain adequate numbers of hits. Two fields from the borderzone- one field from each end of the infarct sector. Two fields from intact myocardium, well separated, but not in conjunction with the infarct zone. Fields with high numbers of cross-sectioned vessels were preferred. We decided to measure the vessels by area. A simple count of the number of vessels in each field would not reflect the dimensions of the vessels. The vessel area should also be indexed with respect to the area of tissue evaluated. The majority of observed vessels in the scar tissue and in the remote myocardium had diameter below 50 μm, and we

assumed it was unlikely that the main beneficial effect of cell therapy would be de novo generation of large blood vessels in these tissues. We therefore excluded vessels with diameter >50 μm from registrations in these tissues, to avioid large random variability in vessel count. A relative area covered by vessel lumina or walls, corrected for the area of tissue evaluated, was then obtained by tissue morphometry with pointcounting on a grid as described for infarct size. With our method, the registered vascular density was low in scar tissue, higher in preserved myocardium and much higher in the borderzone tissue. This finding is well in accordance with the low metabolic demands in scar tissue (resulting from an ischemic injury), and with our definintion of borderzone tissue, which mainly comprised eventual granulation tissue localized between scar tissue and intact myocardium. Granulation tissue is defined by its "granulated" appearance due to high density of small blood vessels. As large vessels, by definition, were not excluded from the vessel count in the borderzone tissue, the definition clearly contributed to the relatively high vascular density counted in our study. However, counting was performed with the same criteria on all sections, and the observer was blinded to treatment allocation by recoding the images, thus, no bias was introduced in the comparison between groups.

Vascular density was higher in scar tissue in the MSC groups than in the control group, but not significantly different between groups in the other tissues. However, the amount of granulation tissue in the borderzone area was higher in the MSC groups. Thus, we can hypothesize that counting of "random" fields from the sections probably would have retrieved higher vascular density overall in MSC treated hearts, in accordance with other studies. This distribution of increased vascular densities makes sense, as an increase of vascular density in intact myocardium should not be expected (there is no physiologic demand), nor lead to improved LV function. The scar tissue may have been generated under the influence of eventual angiogenic paracrine factors, but do not contribute to contractility. An improvement of LV function after cell therapy in AMI should mainly result from the salvage or regeneration of cardiomyocytes. We found an enlarged active borderzone suggesting ongoing reparative processes in a well vascularized environment, with higher probability of reconstitution of contractility than in an established scar tissue. However further identification of the mechanisms behind reduced scar formation and improved LV function was not addressed in our study.

The morphometric approach is well recognized also in quantitative tissue analysis and evaluation of vascularization[76], and the major limitation is the workload. Publications with direct comparison of methods for quantification of vascular density in experimental cardiac

animal studies have, to my knowledge, not been published. We analyzed 6 random sections pr. animal. The number of sections pr. animal was determined by pilot calculations to obtain representative average values for all animals.

Our results in comparison to other studies

As a general comment, different groups use different strains of animals, have different cell clones and use different methods, all factors making a direct quantitative comparison of results difficult to interpret. Our study can be compared with the study by Mazo et al.[96] They compared intramyocardial injections of ADSCs, adipose derived cardiomyogenic cells and mBMCs with placebo. The cells were harvested from GFP+ transgenic mice and injected in rats 1 month after ligation of LAD. Rats were immunosuppressed with cyclosporin A and harvested 1 week and 1 month after injections. GFP positive cells were found in the hearts after 1 week, but not 1 month. Except from the cardiomyogenic cells already expressing troponin at the time of injection, no signs of (trans-) differentiation towards myocardial-, endothelial or smooth muscle lineages occured. Despite this absence of long term engraftment, they observed, for ADSCs vs. placebo, improved LVEF by echocardiography (appx. from 28 to 45 % in the ADSC group vs 30 to 32 % in placebo group), reduced infarct size (11% in the ADSC group vs 20% in placebo group), improved vascular density (appx 50 % more lectin positive vessels pr mm3 in the ADSC group vs the placebo group).

LVEF at baseline was similar with our study. There was an insignificant trend for increase of LVEF in the control group, compared to unhanged LVEF in the control group in our study. In the ADSC group, they found about 15 % increase in LVEF, as compared to 6 % in the SM-MSC group and 4 % in the ADSC group in our study. In Mazo's study, infarct size was 20 % in the placebo group and 11 % in the ADSC group. In our study, infarct size was 26 % in the placebo group and 16 % in both groups receiving MSCs. Thus, cell treated animals had a larger relative effect on infarct size and smaller infarcts at the end of Mazos study. The change in infarct size corresponds well both between studies and with the results by echocardiography. Mazo et. al observed significant increase in vascular density, performing separate stainings for lectin and SMA positive vessels, but only in the infarct zone. The treatment effect on both infarct size, LVEF, and vascular density seem smaller in our study than in Mazos study. This may be explained by less efficient cells or less accurate cell injections, but may also reflect differences in timing of the injections, the animals used or effects of concomitant immunosuppression.

In Mazo's study cells were not retrieved after 4 weeks, and this is in accordance with a few other studies where cells are not identified some time after transplantation. After 1 week, there was no indication that cells were expressing markers suggesting transdifferentiation. In our study, small clusters of cells were found in the infarct zone after 4 weeks, but we did not identify markers from other lineages. This in contrast to several studies reporting long term engraftment of cells and differentiation of MSCs to endothelial cells, smooth muscle cells or even cardiomyocytes.

Cell engraftment depend on a number of factors including cell type, dose, mode of administration, timing after an ischemic event, cell preparation, time elapsed from injection till counting, and also the method for counting (radionuclide markers, MRI, bioluminescence, histological methods), eventual thickness of tissue sections, use of traditional light microscopy vs confocal microscopy and also the sensitivity and specificity of the cell marker used will influence the final number reported.

Regarding differentiation studies, cells with different baseline characteristics have been used. Some of the observations have been made in vitro, cells have been cultured with or without use of growth factors, and some cells have been modified by demethylating agents like 5-azacytidine. Some have injected cells in healthy myocardium, which provide a very different extracellular environment as compared to the infarct zone or the borderzone. It should also be emphasized that expression of single surface markers not necessarily indicate a functional phenotype, and correct interpretation of the microgram and correct specificity of the antibody must be assumed. Autofluorescence and cell fusion are also methodological issues that have been discussed.[20-22, 102]

Our findings of small numbers of engrafted cells, without a significant proportion of cells transdifferentiating during 4 weeks of observation, and a moderate decrease in infarct size, improved LV function and increased vascular density, correspond well with a majority of other publication. To date, there is little evidence supporting that a majority of injected MSCs engraft and differentiate to endothelial cells, SM cells or cardiomyocytes. Cell therapy with MSCs improves LV function, but mechanisms other than cardiomyogenic differentiation of transplanted cells must be involved.

Mechanisms involved in the regenerative process

In theory, the main mechanisms involved in myocardial regeneration by cell therapy after AMI would be:

- generation of cardiomyocytes from the injected cells

- generation of myocardium by mobilization, activation, stimulation and/or chemotaxis of resident or circulating progenitor cells
- myocardial salvage by reduced apoptosis
- improved vascularization, either by differentiation to vascular cells or by stimulation of recipient angiogenesis
- reduced fibrosis

Current evidence support that large numbers of *bona fide* cardiomyocytes in a cell therapy approach can be produced from pluripotent stem cells, either as ESCs[103-105] or eventually from iPS cells[106, 107], but important ethical and methodological issues have to be resolved before human use of these cells can be implemented.[108-110] Although generation of cardiomyocytes from other cell types, including MSCs, have been reported[20, 46], these findings are controversial and, at best, very rare events.[100] In our study, transplanted cells did not differentiate to express neither a cardiomyocyte phenotype nor markers of a smooth muscle or endothelial cell phenotype, and cells were not localized within healthy myocardium nor vessel walls. Nevertheless, MSCs reduced fibrosis or scar formation and improved vascularization without differentiation to vascular cells themselves. The observed improvement in LV function indirectly suggests myocardial salvage and/or generation of myocardium by resident or circulation progenitor cells. Thus, our study supports that several mechanisms are involved, but the study was not designed to identify more detailed mechanisms of action, i.e. identification of soluble factors mediating these effects. However, accumulating evidence support the observation of improved LV function after cell therapy in the absence of differentiation to *de novo* cardiomyocytes. The relative importance of the aforementioned mechanisms is not clear, and these effects may be mediated by one or several soluble factors. Protagonists of the paracrine hypothesis have proposed > 30 putative paracrine factors.[111] Effects on the inflammatory response in ischemia and infarction may also be involved. Myocardial protection after MSC- injections may be mediated by IL-11, Thymosin β4, HGF, IGF-1, VEGF, TSG-6, adrenomedullin, EPO and FGF-2, of which many influence the Akt pathway.[51, 111, 112] Angiogenesis may be mediated by TSG-6, VEGF, nitric oxide, angiopoietin, bFGF, PDGF and MCP-1, all factors shown to be upregulated or released after MSC injections[113-115] Attenuated fibrosis after cardiac MSC therapy has been observed in several studies[50, 116-119], with reduced proliferation of fibroblasts[119] and reduced formation of collagen I and III. Reduced levels of

TIMP 1 and TGF-β are some suggested mediators. [118] MSCs produce TSG-6, shown to inhibit apoptosis and induce angiogenesis.[51] MSCs also, especially when exposed to hypoxic conditions[113], secrete IGF-1, HGF and SDF-1[120], that may attract and stimulate endogenous (cardiac) progentitor cells.[121] Regenerative effects have been demonstrated after injection of merely the supernatant of cultured MSCs, confirming that soluble factors are involved.[122] On the other hand, regenerative effects have been shown to improve significantly if cell survival is enhanced by overexpression of the pro-survival Akt- gene, suggesting that the persisting presence of transplanted cells is important.[123]
Experimental studies have suggested that MSCs have the characteristics needed to regenerate myocardium by all the mechanisms mentioned. However, transdifferentiation of MSCs to express cardiomyocyte markers have only been observed in a few studies, and small numbers of myocytes generated cannot the significant improvement in LVEF. Thus, the process of transdifferentiation need to be better characterized and enhanced to be clinically applicable. The most important paracrine factors must be identified, and for each of the factors, the ideal dosing and spatial and temporal distribution need to be clarified.

Clinical MSC studies

The clinical experience with cardiac MSC therapy is not as extensive as with unfractioned mBMCs, partly because mBMCs had been used in bone marrow transplantations for almost 40 years before the MSCs were coined. In 2004 Chen et al reported a randomized controlled study from China.[124] 69 patients with AMI were randomized to receive intracoronary injections of none marrow derived MSCs or placebo 18 days after the acute event. 48 to 60 x 10^9 cells were injected by stop-flow technique. After 3 months most parameters LV function and remodeling were significantly better in the MSC group. In another phase I cohort study published in 2005, Katritsis et al cultured bone marrow derived MSCs from patients with anteroseptal ST-elevation MI.[125] 2-4 x 10^6 cells were injected 8 to 1560 days after AMI. After 4 months, significant improvement in WMSI and viable segments during dobutamine-echocardiography was observed. In a phase I cohort study on 10 patients with previous coronary artery bypass grafting (CABG) and stable no-option angina, 7.5 x 10^6 mBMCs mixed with 7.5 x 10^6 MSCs were injected intracoronarily.[126] No adverse events were reported, and after 6 months LVEF, ischemia and quality of life was improved. In another Chinese study, patients received intracoronary MSC injections after recanalization and stenting of a chronically occluded LAD. Significant improvement in LVEF and exercise capacity was observed in the MSC group (n=22), but not in the control group (n=23).[127]

The feasibility of intracoronary MSC- injections is controversial, as vascular plugging has been reported. Most ongoing studies use guided catheter systems for transendocardial delivery of cells. In a study by Friis et al, 31 patients with no-option angina were included to receive autologous bone marrow derived MSCs stimulated with vascular endothelium growth factor (VEGF).[128] 10-15 injections were performed to transplant a mean of 21.5 x 10^6 cells. No procedural complications were observed, and after 6 months patients had increased exercise capacity, and reduction in CCS class, angina attacks and nitroglycerine consumption.

The recently published C-CURE trial included patients with heart failure and NYHA class II-III.[129] Bone marrow derived MSCs were treated with growth factors to stimulate cardiomyogenic differentiation of the MSCs. This cocktail had previously been tested in an animal model.[130] In the clinical double blind study (n=45), catheter based injections of cardiopoietic MSCs provided significant improvement in LVEF (5.2±0.6% vs 1.0±0.7%, $p<0.01$) and 6 min walking distance (+52±19 m vs -21±14 m, $p<0.01$) as compared to the control group. Intravenous administration of allogeneic MSCs has also been attempted in one randomized double blind study with 53 included patients.[49] High dose and low dose of the commercial cell product Provacel® was compared to placebo by infusion 1-10 days after reperfused AMI. The primary safety endpoint was met, and provisional efficacy endpoints showed lower occurence of ventricular arrhythmias and a trend for better LV function in the MSC groups.

Thus, most clinical studies report beneficial effects of MSC therapy. However, a variety of modes of administration, cell doses, timing after AMI, subsets of cells and endpoints have been used. Most of the studies are not randomized or double blinded. The number of patients is limited, and no study has been powered to evaluated effects on clinical endpoints. As MSCs are not characterized by a specific phenotypic marker, the cell populations are probably heterogeneous and standardized protocols for cell preparation are warranted.

Lost in translation

For the cell types investigated in both experimental and human studies, ie skeletal myoblasts, mBMCs and MSCs, there is a gap between the substantial regenerative effects reported in some experimental studies and the very modest effects observed in clinical studies. There are several plausible factors explaining observation:

- Dose. Myocardium contains approximately 20 x 10^6 cardiomyocytes per gram of tissue. In our experimental study, the AMI affected 20-30 % of a 0.5-1 g left

ventricle, i.e. < 5 x 10^6 cells were lost. 3 x 10^6 MSCs were injected. In a large human AMI, 20-30 % of a 300-350 g ventricle is lost, i.e 1-1.5 x 10^9 cardiomyocytes.[1, 131, 132] In the REPAIR-AMI study, 236 x 10^6 mBMCs were injected, of which 1.5 % were CD 34 positive, thus containing the fraction of cells with stem cell properties. > 90 % of injected cells are lost within hours.[40] Thus, less than 400.000 stem cells are expected to regenerate 1-1.5 x 10^9 cardiomyocytes.

- Size. Cells are injected to engraft in ischemic or infarcted tissue. The vascular supply is impaired. The volume of the infarct zone is 300 times larger in humans than in rats The murine left ventricle has a wall thickness of about 1 mm, in comparison to the human heart with an end-diastolic LV wall thickness normally within the range 7-11 mm. Based on simple geometric assumptions, adequate layers of transplanted cells have to be at least 10-fold thicker in humans than in rats. The same maximum distance for passive diffusion of oxygen and nutritients to cells will apply in the two species, suggesting that the likelihood for survival of an adequate number of cells after simple injection will decrease with the size of the subject.
- Properties of the cell product. It has been documented that migratory and proliferative properties of progenitor cells are progressively impared depending of the occurence of cardiovascular risk factors like age, diabetes and smoking. Thus, autologous cells harvested from patients in the population suffering from postinfarction heart failure do not have the same regenerative potential as progenitor cells from young and healthy individuals (animals or humans).[133]
- Interspecies variations. Several differences in physiology between species are known.[134] Mice have a metabolic rate (pr. weight) 3 times higher than men. Cancer risk in laboratory mice is substantially higher than in man, suggesting genetic instability. In the newt, spontaneous regeneration of heart and limbs occur after injury. Although the capacity for spontaneous myocardial regeneration in mice seem to be lost within 1 week after birth[135], interspecies differences in regenerative processes are not fully explored.

Conclusions

In patients from the ASTAMI study, mBMC therapy did not significantly influence clinical events, left ventricular function, infarct size, exercise capacity or quality of life during 3 years follow up. The conclusion is limited by the moderate number of patients included in the study. However, the result is supported by accumulating evidence from other trials suggesting, at best, moderate improvement in LVEF after mBMC injections in AMI. The effect of mBMC therapy on patient survival has not yet been addressed in an adequately designed clinical randomized trial, and the optimal cell dose, timing, method for administration and mechanisms of action are not clarified.

Human derived MSCs from skeletal muscle (SM-MSCs) and adipose tissue (ADSCs) improve LV function when injected intramyocardially in nude rats 1 week after after permanent ligation of LAD. The improvement in LV function is related to an observed reduction in infarct size, increase in granulation tissue in the borderzone, and increase vascular density observed in animals receiving MSC injections. Small clusters of MSCs are observed in the scar tissue and borderzone after 4 weeks. No transdifferentiation to cardiomyocytes, endothelial cells or smooth muscle is observed, suggesting that that the MSCs contribute to LV regeneration by other mechanisms, like stimulation of blood vessel formation and modulation of scar formation.

Limitations

Detailed methodology in cardiac MRI, quality-of-life analyses, cell harvesting and -processing are not part of the present thesis. In the overview of experimental and clinical cell therapy in heart disease, studies using bone marrow cells and mesenchymal stem cells after acute myocardial infarction are emphasized.

Future perspectives

At present, the evidence does not justify implementation of cardiac cell therapy in routine clinical practice. True regeneration of cardiac tissue to restore cardiac systolic function will require further progress in research. The technique of induced pluripotency in autologous cells (iPS-cells)[136] may circumvent the ethical and immunological issues hampering the use of ESCs. However, iPS cells can be genetically and epigenetically heterogeneous, and there will be a tug of war between pluripotency and potential for proliferation versus lineage commitment and differentiation control.[137-139] Non-genomic reprogramming and further insight in the signaling pathways of differentiation will increase efficacy and safety.[140] Tissue engineering with patches containing vascular- and myogenic cells in a scaffold of biomaterials may promote engraftment and survival of sufficient numbers of contractile cells.[141-143] However, a single cell type and and one single application will not cover all potential indications for cardiac cell therapy. In acute settings, in vitro culture of cells and iPS- based tissue engineering will be too time consuming, and in no-option angina, angiogenesis to support already existing cardiomyocytes is preferable. In these applications, adult stem cells may still serve as the best available agents, but there will be a search for adult cell types with higher regenerative and/or angiogenic potential to replace the use of unfractioned mBMCs. The paracrine mechanisms will hopefully be defined, and recombinant factors may replace cell therapy in some applications. Clearly, there is a clinical demand for efficient cardiac regeneration, and cell therapy has a great potential, conceptually. Convincing results from properly designed and conducted clinical trials will, however, be pivotal for future clinical use.

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I

II

III

Errata:

For printing, a new page 2 was added, and further page numbers changed accordingly

Page 7 line 11; ”left ventricular” has been added

Page 10 line 25; “two-stem” has been changed to “two stem”

Page 11 line14; “has” has been changed to “have”

Page 13 line 34; “injection” has been changed to “ injections”

Page 27 line 2; “3 short axis cineloops” has been changed to “2 short…”

Page 27 line 4-7; figure 1, 2 and 3 have been changed to figure 2,3a and 3b, and references to figure numbers (on page 27 and 28) have been corrected accordingly.

Page 38 line 30; “when new” has been changed to “when a new”

Page 40 line 2; “demonstrate” has been changed to “demonstrates”

Page 42 line 28; “(REF)” has been removed

Page43 line 14; “in” has been added

Page 47 line 7; one “.” Has been removed.

Page 72 line 39; reference 120 has been updated.